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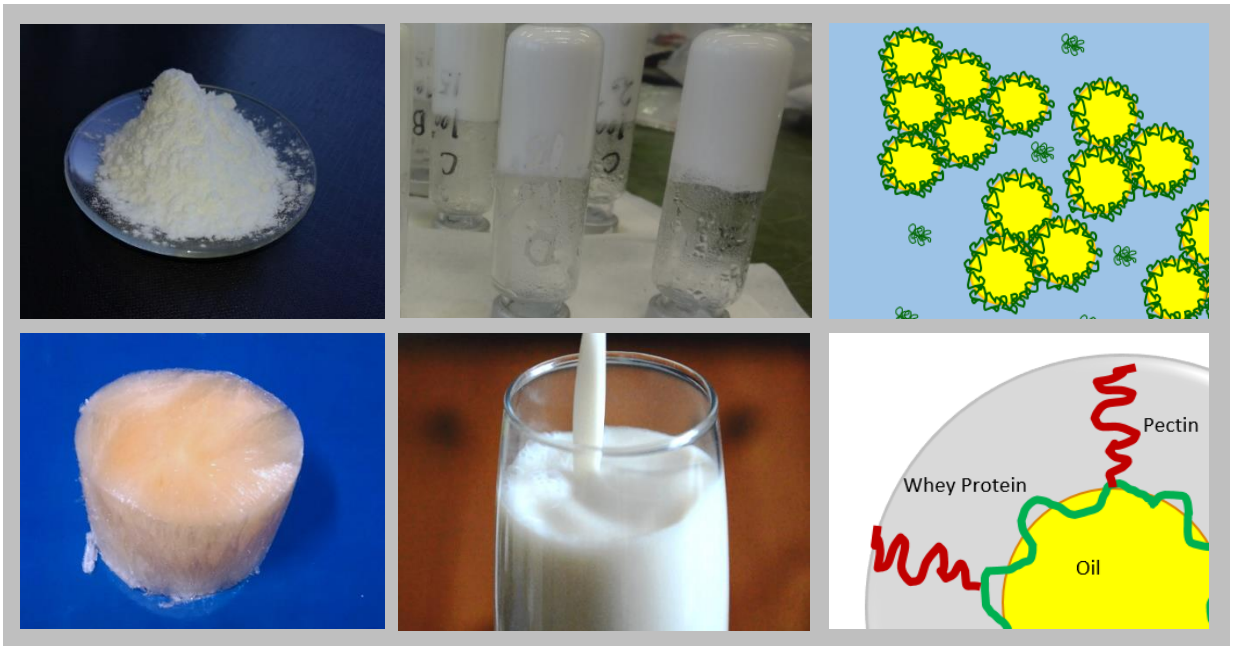

EMULSIFYING AND STABILIZING PROPERTIES OF WHEY PROTEIN - PECTIN CONJUGATES PREPARED BY DRY HEAT TREATMENT

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LIST OF ABBREVIATIONS AND SYMBOLS

ANOVA	Analysis of Variance
BSA	Bovine Serum Albumin
BV	Biological value
d3,2	Sauter mean diameter of oil droplets
d4,3	Volume-weighted average diameter of oil droplets
g	Gram
HMP	High Methoxyl Pectin
kDa	Kilo Dalton
LMP	Low Methoxyl Pectin
M	Molar
m.Pa.s	Milli pascal.second
min	Minute
mL	Milliliter
N	Normality
NMR	Nuclear Magnetic Resonance
O/W	Oil in Water
PAGE	Polyacrylamide Gel Electrophoresis
pfg	Pulsed Field Gradient
ppm	Parts per million
QCM-D	Quartz Crystal Microbalance equipped with Dissipation
SDS	Sodium Dodecyl Sulfate
WPC	Whey Protein Concentrate
WPI	Whey Protein Isolate
Γ	Protein load
γ	Shear rate
θ	Ellipticity
μ	Apparent viscosity
μm	Micrometer
ρ	Density
τ	Shear stress

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SAMENVATTING

Een deel van de eiwitten in melk bestaan uit wei-eiwitten. De belangstelling in deze wei-eiwitten is nog steeds groot omwille van hun verschillende functionaliteiten. Hun toepassing is echter beperkt door hun hitte-instabiliteit. Wei-eiwitten blootstellen aan een temperatuur boven hun denaturatietemperatuur induceert denaturatie van de eiwitten en aggregatie. Daardoor verliezen melkweiproteïnen hun oplosbaarheid, wat leidt tot de vermindering van hun functionaliteit.

In hoofdstuk 1 worden de eigenschappen van melkweiproteïnen, zoals hun samenstelling en functionaliteit, in meer detail besproken. In dit hoofdstuk 1 ligt de focus op de hittestabiliteit van wei-eiwitten. In dit eerste hoofdstuk worden ook de processen besproken die betrokken zijn in de hittedenaturatie en aggregatie van eiwitten. Sommige factoren die de hittestabiliteit van melkweiproteïnen beïnvloeden, zoals temperatuur, pH en zoutgehalte, worden ook behandeld. Melkwei-eiwitten zijn erom bekend een goede emulgerende werking te hebben. Daarom wordt ook het effect van hittebehandeling op de stabiliteit van emulsies, gestabiliseerd met melkwei-eiwitten, besproken. Verschillende methoden ter verbetering van de hittestabiliteit van eiwitten worden voorgesteld. Onder de beschikbare methoden was onze studie gericht op de chemische modificatie van melkweiproteïnen door interactie met andere biopolymeren, namelijk polysachariden, via een Maillard type reactie. Deze methode omvat het mengen van eiwitten en polysachariden, gevolgd door droge hittebehandeling van het gedroogde mengsel gedurende een bepaalde tijd bij een verhoogde temperatuur en constante relatieve vochtigheid. In deze studie werd pectine gekozen als het polysacharide dat geconjugeerd (chemisch gekoppeld) werd met melkwei-eiwitten. De aanwezigheid van pectine zou naar verwachting bijdragen aan de stabilisatie van de melkwei-eiwitten tegen thermische denaturatie door middel van sterische stabilisatie. Een gedetailleerde beschrijving van de chemische koppeling van eiwitten en polysachariden door middel van Maillardreacties wordt ook in dit hoofdstuk gegeven, evenals de factoren die het proces beïnvloeden.

Hoofdstuk 2 beschrijft de conjugatie van weiproteïne-isolaat (WPI) en laag methoxyl pectine (LMP). De WPI-LMP-conjugaten werden bereid door de WPI- en LMP-oplossingen in een respektievelijke verhouding van 1:0, 4:1, 2:1 en 1:1 te mengen. De mengsels werden

gevriesdroogd en in droge toestand behandeld bij 60°C gedurende 0, 4, 8 en 16 dagen. Karakterisering en bevestigingstests werden uitgevoerd om de vorming van de conjugaten te evalueren. Bovendien werd de hittestabiliteit van de conjugaten ook getest door verwarming van de WPI-LMP-conjugaatoplossingen bij 80°C. De vorming van de conjugaten bij droge hittebehandeling van de WPI-LMP-mengsels werd bevestigd met behulp van SDS-PAGE, TNBS analyse en bruine pigmentvorming. Uit de resultaten bleek dat bij droge hittebehandeling nieuwe verbindingen met een hoge moleculaire massa werden gevormd. Bij het verwarmen van de WPI-LMP-conjugaatoplossingen bleek dat de eiwitoplosbaarheid van WPI opvallend verbeterde nadat het met LMP bij alle WPI-LMP-verhoudingen was geconjugeerd. Dit fenomeen werd niet waargenomen als de mengsels van WPI en LMP niet droog bij verhoogde temperatuur behandeld werden. Dit werd toegeschreven aan de aanwezigheid van een sterke sterische kracht die door de bijgevoegde LMP wordt geleverd. Door de aanwezigheid van covalent gebonden LMP werd de aggregatie van eiwitten tijdens verhitting voorkomen. Bovendien biedt pectine extra hydrofiele groepen aan die de oplosbaarheid van de melkwei-eiwitten verbeteren.

De functionaliteit van de in hoofdstuk 2 gevormde conjugaten werd vervolgens geëvalueerd in een olie-in-water emulsiesysteem. 10% olie-in-water emulsies gestabiliseerd door 0,5% WPI, 0,5% WPI-LMP mengsel en 0,5% WPI-LMP conjugaten werden bereid. De WPI-LMP-conjugaten werden bereid in een verhouding van 2:1 en werden droog-hittebehandeld bij 60°C en een relatieve vochtigheid van 74% voor 0, 1, 2, 3, 4, 8 en 16 dagen. De emulgerende activiteit van de conjugaten en de hittestabiliteit van de emulsies die door de conjugaten werden gestabiliseerd, werden onderzocht bij een pH van 6,5 en een pH van 5,0. Deze laatste werd uitgevoerd in aan- en afwezigheid van een lage concentratie NaCl. Bovendien werd de invloed van de pectine-concentratie onderzocht. Voor dit doel werden WPI-LMP-conjugaten bereid bij vier verschillende WPI- ten opzichte van LMP-verhoudingen, namelijk in de verhouding 1:0, 4:1, 2:1 en 1:1.

In hoofdstuk 3 worden de resultaten van deze studie weergegeven. Op basis van de resultaten werd geconstateerd dat de WPI-LMP conjugaten een grotere emulgerende activiteit vertoonden dan het oorspronkelijke WPI en de (niet-geconjugeerde) WPI-LMP mengsels. Het verwarmen van de emulsies bij 80 en 120°C onthulde dat de emulsies die door de WPI-LMP-conjugaten werden gestabiliseerd, zeer hittestabiel bleken. De emulsies waren in staat hun

initiële druppelgrootte, viscositeit en zelfs oproomstabiliteit te behouden na verwarming. Zonder droge hittebehandeling waren WPI-LMP-mengsels in staat om emulsies tegen hitte te stabiliseren bij pH 5,0, maar niet wanneer 30 mM zout aan de emulsies werd toegevoegd. De stabiliteit van emulsies bij pH 5,0, die door WPI-LMP-conjugaten werden gestabiliseerd, werd toegeschreven aan de verschuiving van het IEP van WPI naar een lagere pH-waarde bij droge hittebehandeling met LMP. Wat de invloed van pectineconcentratie betreft, bleek dat zelfs bij een lage pectineconcentratie de WPI-LMP conjugaten al een uitstekende hittestabiliteit vertoonden, terwijl een hogere pectineconcentratie nodig was om de emulsies te stabiliseren tegen hitte wanneer de WPI-LMP mengsels niet droog-hittebehandeld werden. De algemene resultaten in deze studie impliceerden dat de aard van de interactie tussen WPI en LMP belangrijk was voor de stabiliserende activiteit van de conjugaten.

In hoofdstuk 4 wordt de studie beschreven die werd uitgevoerd over de adsorptie van WPI-, WPI-LMP-mengsels en WPI-LMP-conjugaten via een kwartskristalmicrobalans met dissipatiemeting (QCM-D). De resultaten toonden aan dat WPI een visco-elastische laag opleverde die de oliedruppels stabiliseerde tegen druppelflocculatie. Deze geadsorbeerde laag viel uiteen en werd rigider toen deze werd blootgesteld aan een lage pH (pH 5,0). Bij conjugatie van WPI en LMP door droge hittebehandeling werd de laag meer visco-elastisch en dikker door de aanwezigheid van covalent gebonden LMP. Een soortgelijke laag werd ook gevormd door elektrostatische interactie van WPI met LMP. De aanwezigheid van deze dikke en visco-elastische laag verklaarde de stabiliteit van de emulsies die gestabiliseerd werden door de WPI-LMP-conjugaten en WPI-LMP-complexen die werden gevormd via elektrostatische interactie. Deze resultaten ondersteunen de bevindingen in het vorige hoofdstuk waarin werd opgemerkt dat de WPI-LMP conjugaat gestabiliseerde emulsies een betere stabiliteit hadden dan de WPI gestabiliseerde emulsies. De dikte van de geadsorbeerde laag en de massa van de geadsorbeerde eiwitten werd ook succesvol bepaald met behulp van QCM-D.

Hoofdstuk 5 behandelt de studie van WPI-LMP conjugaten via pulsed-field gradient-nuclear magnetic resonance (pfg-NMR). In dit deel werd een poging gedaan om de hoeveelheid gereageerde WPI en vrije WPI in de WPI-LMP-conjugaten te kwantificeren. Dit deel van de studie is belangrijk om een beter inzicht te krijgen in de emulgerende activiteit en de hittestabiliteit van de conjugaten, evenals over de efficiëntie van de droge hittebehandeling.

Het resultaat van droge hittebehandeling werd bepaald met behulp van pfg-NMR als functie van de droge hittebehandelingstijd en de pectineconcentratie. Verder werd ook de graad van elektrostatische interactie in de WPI-LMP complexen bij pH 5,0, 5,5 en 7,2 bepaald. Uit de resultaten blijkt dat langere incubatietijden en hogere pectineconcentraties de hoeveelheid gereageerde WPI verhogen. Elektrostatische interactie tussen WPI en LMP bij pH 5,0 resulteerde ook in een hogere hoeveelheid complexen dan bij pH 5,5 en pH 7,2. Gezien de resultaten waargenomen in hoofdstuk 3, kan geconcludeerd worden dat de emulgerende activiteit van de WPI-LMP-conjugaten werd beïnvloed door de opbrengst van het droge hittebehandelingsproces. Anderzijds werd de hittestabiliteit van de conjugaten minder beïnvloed door de opbrengst. Dit werd ondersteund door het feit dat zelfs met een lagere opbrengstwaarde of een lagere mate van conjugatie, WPI-LMP-conjugaten al een grote stabiliteit tegen hitte vertoonden. Door deze studie werd aangetoond dat pfg-NMR een veelbelovend hulpmiddel is om de interactie tussen eiwitten en polysachariden te bestuderen. Bovendien is pfg-NMR sensitief en relatief minder arbeidsintensief in vergelijking met de methoden die de fysieke scheiding van gereageerde en niet-gereageerde eiwitten vereisen.

Het is bekend dat de functionaliteit van pectines wordt beïnvloed door hun veresteringsgraad. Hoofdstuk 6, het laatste experimenteel deel van dit proefschrift, geeft informatie over de invloed van de veresteringsgraad op de prestatie van de WPI-pectine conjugaten. Twee verschillende WPI-pectine conjugaten werden bereid, namelijk WPI-LMP conjugaten en WPI-HMP (hoog methoxyl pectine) conjugaten. De emulgerende activiteit en hittestabiliteit van deze conjugaten werden onderzocht bij pH 6,5 en pH 5,0. Vóór de bereiding van de WPI-pectine conjugaten werden de pectine-oplossingen gedialyseerd met Milli-Q® water ter verwijdering van dextrose en sucrose uit het gebruikte HMP en LMP, om de invloed van deze aanwezige suikers in de pectine te elimineren of te minimaliseren. Deze suikers met een laag moleculaire massa werden door de fabrikant aan de pectine toegevoegd om de functionaliteit van de pectine te standaardiseren en om de oplosbaarheid van de pectine te verbeteren. Er werd gevonden dat de veresteringsgraad geen invloed had op de emulgerende activiteit en de hittestabiliteit van de emulsies. Anderzijds bleek de veresteringsgraad de oproomstabiliteit van de emulsies te beïnvloeden. WPI-pectine conjugaten bereid met HMP hadden een betere oproomstabiliteit dan die bereid met LMP.

In hoofdstuk 7 worden de algemene conclusies gepresenteerd die werden verkregen uit de hele doctoraatsstudie. De algemene resultaten geven aan dat de vorming van WPI-pectine conjugaten via droge hittebehandeling een veelbelovende methode is om de hittestabiliteit van weiproteïnen te verbeteren. Bovendien verruimt het ook de toepassingsmogelijkheden van weiproteïnen in de voedingsindustrie. Samen met de algemene conclusie worden ook mogelijkheden voor verder onderzoek voorgesteld.

SUMMARY

Whey proteins are milk proteins which have been well studied. Whey proteins are famous due to their various functionalities. However, their application is limited by their low heat stability. Exposing whey proteins to a temperature above their denaturation temperature induces protein denaturation and aggregation. As a consequence, whey proteins lose their solubility which leads to the reduction of their functionality. In Chapter 1, the properties of whey proteins, such as composition and functionality, are discussed in more detail. The focus of this chapter was laid on the heat stability of whey proteins. A description of the processes involves in heat denaturation and aggregation of proteins can be found in this chapter. Some factors which influence the heat stability of whey proteins, such as temperature, pH, and electrolytes, are also elaborated. Whey proteins are known for having a good emulsifying activity. Thus, the effect of heat treatment on the stability of whey protein stabilized emulsions is also discussed. Several methods to improve the heat stability of whey proteins are proposed. Among the available methods, our study was focused on the chemical modification of whey proteins upon interaction with other biopolymers, i.e.: polysaccharides, via a Maillard type reaction. This method involves mixing of protein and polysaccharides, followed by dry heat treatment of the dried mixture at an elevated temperature and constant relative humidity for a given time. In this study, pectin was chosen as the polysaccharides to be conjugated with whey proteins. The presence of pectin was expected to contribute to the stabilization of the whey proteins against thermal denaturation through steric stabilization. A detailed description of the conjugation of proteins and polysaccharides through Maillard type reactions is also provided in this chapter, as well as the factors influencing the process.

Chapter 2, describes the conjugation of protein–polysaccharide using whey protein isolate (WPI) and low methoxyl pectin (LMP). The WPI-LMP conjugates were prepared by mixing WPI and LMP solutions at a WPI to LMP ratio of 1:0, 4:1, 2:1, and 1:1. The mixtures were lyophilized and dry heat treated at 60^{o}C and a relative humidity (RH) of 74% for 0, 4, 8, and 16 days. Characterization and confirmation tests were performed to evaluate the formation of the conjugates. Furthermore, the heat stability of the conjugates was also tested by heating the WPI-LMP conjugate solutions at 80^{o}C. The formation of the conjugates upon dry heat treatment of the WPI-LMP mixtures was confirmed using SDS-PAGE, TNBS analysis, and brown

pigment formation. The results revealed that upon dry heat treatment, new compounds with a high molecular weight were formed. Upon heating the WPI-LMP conjugate solutions, it was found that the protein solubility of the WPI improved remarkably after it was conjugated with LMP at all WPI to LMP ratios. This phenomenon was not observed when the mixtures of WPI and LMP were not dry heat treated. This was attributed to the presence of a strong steric force provided by the attached LMP. Due to the presence of covalently bound LMP, the aggregation of proteins during heating was prevented. Furthermore, pectin provides additional hydrophilic groups which improved the solubility of the whey proteins.

The functionality of the conjugates described in Chapter 2 was then evaluated in an oil in water emulsion system. 10% of oil in water emulsions stabilized by 0.5% WPI, 0.5% WPI-LMP mixture, and 0.5% WPI-LMP conjugates were prepared. The WPI-LMP conjugates were prepared at a ratio of 2:1 and were dry heat treated at 60^{o}C and RH of 74% for 0, 1, 2, 3, 4, 8, and 16 days. The emulsifying activity of the conjugates and the heat stability of the emulsions stabilized by the conjugates were studied at pH 6.5 and pH 5.0. The latter was performed in the presence and absence of a low concentration of NaCl. Furthermore the influence of pectin concentration in the conjugates was also investigated. For this purpose, the WPI-LMP conjugates were prepared at four different WPI to LMP ratios, namely ratio 1:0, 4:1, 2:1, and 1:1. The results of this study are presented in Chapter 3. Based on the results, it was observed that the WPI-LMP conjugates exhibited a greater emulsifying activity than native WPI and mixtures of WPI-LMP. Heating the emulsions at 80 and 120^{o}C revealed that the emulsions stabilized by the WPI-LMP conjugates were highly stable against heat. At both pH conditions, the emulsions were able to retain their initial droplet size, viscosity, and even creaming stability after heating. Without dry heat treatment, WPI-LMP mixtures were able to stabilize emulsions against heat at pH 5.0 but failed to provide the same result when 30 mM of NaCl was added to the emulsions. The stability of emulsions stabilized by WPI-LMP conjugates at pH 5.0 was attributed to the shift of the IEP of WPI to a lower pH value upon dry heat treatment with LMP. As for the influence of pectin concentration, it was found that even at low pectin concentration, the WPI-LMP conjugates already exhibited an excellent heat stability, whereas a higher concentration of pectin was needed to stabilize the emulsions against heat when the WPI-LMP mixtures were not dry heat treated. The general results in

this study implied that the nature of the interaction between WPI and LMP was important for the stabilizing activity of the conjugates.

In Chapter 4, a study on the adsorption of WPI, WPI-LMP mixtures, and WPI-LMP conjugates via a Quartz Crystal Microbalance equipped with Dissipation (QCM-D) is described. The results showed that WPI provided a viscoelastic layer which stabilized the oil droplets against droplet flocculation. This adsorbed layer collapsed and became more rigid when it was exposed to a low pH (pH 5.0). Upon conjugation of WPI and LMP through dry heat treatment, the layer became much more viscoelastic and thicker due to the presence of covalently linked LMP. A similar layer was also formed when WPI interacted with LMP via electrostatic interaction. The presence of this thick and viscoelastic layer explained the stability of the emulsions stabilized by the WPI-LMP conjugates and WPI-LMP complexes formed via electrostatic interaction. These results supported the findings in the previous chapter in which it was observed that the WPI-LMP conjugate stabilized emulsions had a better stability than the WPI stabilized emulsions. The thickness of the adsorbed layer and the mass of the adsorbed proteins was also successfully determined using QCM-D.

Chapter 5 deals with the study of WPI-LMP conjugates via pfg-NMR. In this part, an attempt to quantify the amount of reacted WPI and free WPI in the WPI-LMP conjugates was made. This part of the study is important for having a better understanding of the emulsifying activity and heat stability of the conjugates as well as on of the efficiency of the dry heat treatment. The yield of dry heat treatment was determined using pfg-NMR as a function of dry heat treatment time and pectin concentration. Furthermore, the yield of WPI-LMP interaction via electrostatic interaction at pH 5.0, 5.5, and 7.2 was also determined. The results indicated that a longer incubation times and higher pectin concentrations increased the amount of reacted WPI. Electrostatic interaction between WPI and LMP at pH 5.0 also yielded a higher amount of complexes than at pH 5.5 and 7.2. Considering the results observed in Chapter 3, it can be concluded that the emulsifying activity of the WPI-LMP conjugates was influenced by the yield of the dry heat treatment process. On the other hand, the heat stability of the conjugates was less influenced by the yield. This was supported by the fact that even with a lower yield value or lower degree of conjugation, WPI-LMP conjugates already exhibited a great stability against heat. Through this study, it was proven that pfg-NMR is a promising tool to study the interaction between proteins and polysaccharides. Moreover, pfg-NMR is sensitive and

relatively less labour intensive as compared to the methods that require the physical separation of reacted and unreacted proteins.

It is known that the functionality of pectins is influenced by their degree of esterification (DE). Chapter 6, the last part of this dissertation, provides information about the impact of DE on the performance of the WPI-pectin conjugates. Two different WPI-pectin conjugates were prepared, namely WPI-LMP conjugates and WPI-HMP (high methoxyl pectin) conjugates. The emulsifying activity and heat stability of these conjugates were investigated at pH 6.5 and pH 5.0. Prior to the preparation of the WPI-pectin conjugates, the pectin solutions were dialyzed against Milli-Q water to remove dextrose and sucrose from the HMP and LMP used, respectively, in order to eliminate or minimize the influence of the sugar present in the pectin. These low molecular weight sugars were added to the pectin by the manufacturer to standardize the functionality and help enhance the dissolution of the pectin. It was found that the DE did not influence the emulsifying activity and heat stability of the emulsions. Nevertheless, the DE seemed to influence the creaming stability of the emulsions. WPI-pectin conjugates prepared with HMP had a better creaming stability than those prepared with LMP.

In Chapter 7, the general conclusions obtained from the whole doctoral studies are presented. The general results indicate that the formation of WPI-pectin conjugates via dry heat treatment is a promising method to improve the heat stability of whey proteins. Furthermore, it also broadens the application of whey proteins in food industries. Along with the general conclusion, the possibilities for further research are also proposed.

OUTLINE OF THE RESEARCH

The content of the dissertation is divided into 7 main chapters. The first chapter (Chapter 1) focuses on the literature study of the heat stability of whey proteins and factors influencing their heat stability. Furthermore, several methods to improve the heat stability of whey proteins were discussed. In addition, the objective of this dissertation was briefly explained at the end of this chapter.

Chapter 2 deals with the formation of the whey protein isolate (WPI)-low methoxyl pectin (LMP) conjugates to improve the heat stability of whey proteins in solutions. In this part, the influence of dry heating time and WPI to LMP ratio was discussed. The formation of the WPI-LMP conjugates upon dry heating was confirmed by several tests, such as TNBS measurement, SDS-PAGE analysis, colour, pH, and NMR measurements. Afterwards, the effect of heat treatment on the solubility of WPI was evaluated, as protein solubility is essential for the functionality of WPI.

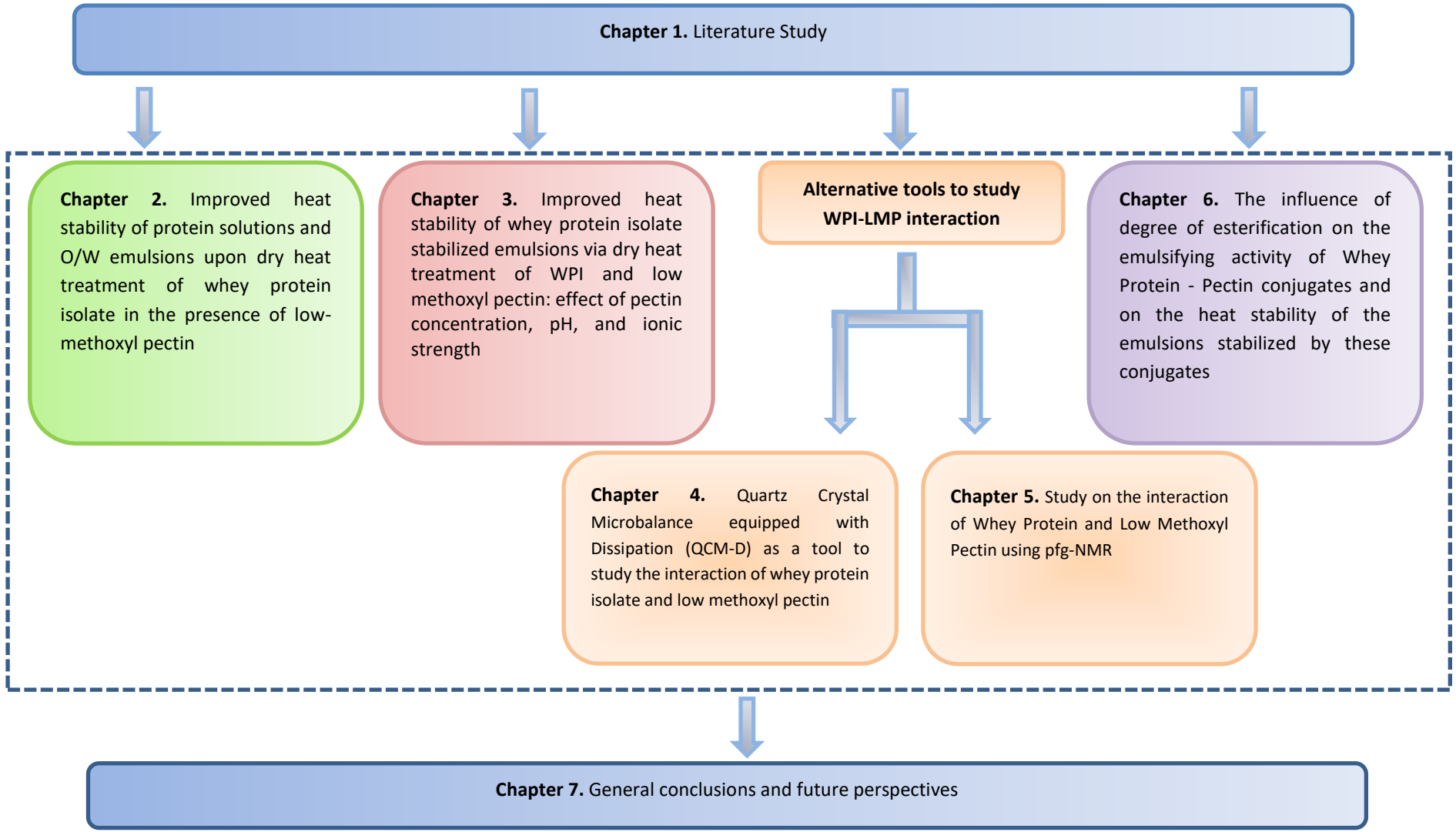
Chapter 3 focuses on the application of WPI-LMP conjugates in an oil-in-water emulsion system. Several factors, such as pectin concentration, pH, and ionic strength of the emulsions, which influence the emulsifying activity of the conjugates and heat stability of the emulsions stabilized by the conjugates were discussed. The emulsifying activity of the conjugates and heat stability of the emulsions were evaluated by performing particle size, viscosity, creaming velocity, electrophoretic mobility, and protein adsorption measurements.

Chapter 4 and 5 present an alternative method to study the interaction of WPI and Pectin. The study of WPI-LMP interaction using a Quartz Crystal Microbalance equipped with Dissipation (QCM-D) was discussed in Chapter 4. Chapter 5 is devoted to study and quantify the interaction of WPI and LMP using high resolution NMR (nuclear magnetic resonance). The influence of pH, pectin concentration, and dry heat treatment on the diffusion coefficient and yield of the dry heat treatment was discussed in Chapter 5.

Chapter 6 describes the influence of the degree of esterification on the performance of WPI-pectin conjugates. Two different types of pectin, namely high methoxyl and low methoxyl

pectin, were used to prepare WPI-pectin conjugates. Following the conjugates preparation, the emulsifying activity and heat stabilizing activity of the conjugates were evaluated.

To conclude, the general conclusions and future perspective are presented in Chapter 7.


Chapter 1. Literature Study
Chapter 2. Improved heat stability of protein solutions and O/W emulsions upon dry heat treatment of whey protein isolate in the presence of low-methoxyl pectin
Chapter 3. Improved heat stability of whey protein isolate stabilized emulsions via dry heat treatment of WPI and low methoxyl pectin: effect of pectin concentration, pH, and ionic strength
Alternative tools to study WPI-LMP interaction
Chapter 6. The influence of degree of esterification on the emulsifying activity of Whey Protein - Pectin conjugates and on the heat stability of the emulsions stabilized by these conjugates
Chapter 4. Quartz Crystal Microbalance equipped with Dissipation (QCM-D) as a tool to study the interaction of whey protein isolate and low methoxyl pectin
Chapter 5. Study on the interaction of Whey Protein and Low Methoxyl Pectin using pfg-NMR
Chapter 7. General conclusions and future perspectives

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Dairy proteins are one of the main sources of proteins due to their nutritional value, unique properties, and technological importance (Fox, 2009; Zayas, 1997b). Caseins and whey proteins are two major groups of milk proteins which have been studied intensively. Casein is obtained after precipitation at pH 4.6 and exists as a colloidally dispersed micelle with high nutrient value and functionality (Zayas, 1997b). In this study, the focus was given to the whey protein fraction, the second most known group of milk proteins which is widely known due to its diverse functional properties and nutrient value. Due to this reason, whey proteins have been used in different food products including sport beverages, liquid meat replacements, ice cream, salad dressing, bakery products, infant foods and various dairy products (Fitzsimons, Mulvihill, & Morris, 2007; Jovanović, Barać, & Maćej, 2005).

Heat treatment such as pasteurization and sterilization is normally used to prolong the shelf life of food products. Despite having an excellent functionality, whey proteins are very susceptible to heat induced denaturation and polymerization which leads to the reduction of their solubility and functionality (Kessler & Beyer, 1991; Lee, Morr, & Ha, 1992). Furthermore, heat induced denaturation and aggregation alter the quality of the products containing whey proteins which is highly undesirable. Due to its heat lability, protein aggregation takes place during heating, which increase the droplet size and viscosity of the emulsions stabilized by whey proteins. This phenomenon becomes the biggest challenge of whey protein applications in food industry since heat treatment is almost inevitable. In order to broaden the application of whey proteins, it is of utmost importance to improve the heat stability of whey proteins. Whey proteins should be able to maintain their solubility and functionality after heat treatment. Furthermore, there should not be any change in the quality of the products after heat treatment.

In this part of the study, the properties of whey proteins are discussed. The focus will be given to the heat stability of whey proteins as well as factors influencing the heat stability of whey proteins. The effect of heat induced denaturation on protein stabilized emulsions is also discussed. A method to tackle the problem and improve the heat stability of whey proteins is also proposed in the end of this chapter.

1.2 Whey proteins

Whey is a byproduct of the cheese and dairy industry (Zayas, 1997b). The production of whey proteins has been projected to continuously grow in the future. It is either processed further into whey protein isolate (WPI) or whey protein concentrate (WPC) (Brncic, et al., 2009). WPC with protein concentration of up to 75% is produced using a combination of commercial scale ultrafiltration and diafiltration (Morr & Ha, 1993). On the other hand, WPI, which contains a higher amount of protein (±90%), is produced by subjecting whey to an ion exchange process. The proteins adsorbed to the ion exchanger are then desorbed and eluted from the ion exchanger (Goodall, Grandison, Jauregi, & Price, 2008). These proteins are then concentrated and dried using ultrafiltration and spray drying, respectively (Morr, et al., 1993). Proteins produced using ultrafiltration undergo very limited denaturation (Zayas, 1997a).

Whey proteins are recognized as nutritionally superior to other common dietary proteins due to their high amount of essential amino acids, branched chain and sulphur amino acids, as well as their high biological value (BV) and digestibility compared to other protein sources (Smithers, 2008). Moreover, whey proteins contain biologically active proteins and peptides which are suitable for medicinal applications. As an example, evidences about anti-cancer effects of lactoferrin, lactoperoxidase and serum albumin have been reported in some studies (Bounous, Batist, & Gold, 1991; Gill & Cross, 2000).

Due to their diverse functionality, whey proteins have been widely used as ingredients in different types of traditional and novel foods (Kinsella & Whitehead, 1989). The functional properties of whey proteins include gelling (Alting, Hamer, de Kruif, & Visschers, 2000; Dickinson & Euston, 1991), emulsification (Damodaran, 2005; Demetriades, Coupland, & McClements, 1997b; Gunasekaran, Ko, & Xiao, 2007), foaming (Damodaran, 2005; Murray, Durga, Yusoff, & Stoyanov, 2011) and water binding. As a gelling agent, WPI is useful for designing and improving the textural properties of various foods, such as dairy, meat and bakery products (Jovanović, et al., 2005). Based on how the gels are prepared, they can be divided into heat induced gels and cold set gels (Jovanović, et al., 2005). It also works well in stabilizing foams (Damodaran, 2005). Whey proteins are highly soluble in an aqueous phase and are able to diffuse rapidly at the interface reducing the interfacial tension between air and water which is then followed by unfolding and formation of an interfacial film around the

air bubbles which stabilizes them (Kinsella, 1981). Among the functional properties of whey proteins, its role as a natural emulsifier has been widely recognized. Due to their amphiphilic nature, whey proteins are often used to stabilize emulsions, especially oil in water emulsions (Kato, Mifuru, Matsudomi, & Kobayashi, 1992). Upon emulsification, whey proteins rearrange their structure so that the hydrophobic part is anchored in the oil phase while the hydrophilic groups is in the aqueous part leading to the stabilization of the oil droplets (Dickinson, 1999).

The functionalities of whey proteins are highly influenced by several factors such as the intrinsic physiological properties of the native proteins, their amino acid composition and their sequence, the ratio of hydrophobicity to hydrophilicity, charge distribution, and flexibility (Kinsella, et al., 1989; Zayas, 1997b). In addition, the external factors such as processing conditions applied to obtain whey proteins, isolation methods, protein content, pH, temperature and ionic strength, as well as the interaction with other food ingredients influence the functional properties of whey proteins by changing the conformation of whey proteins (Kinsella, et al., 1989; Zayas, 1997b).

Whey proteins consist of α-lactalbumin, β-lactoglobulin, immunoglobulins, and serum albumin (BSA), which constitute 22%, 60%, 9% and 5.5% of the total whey proteins, respectively (Bryant & McClements, 1998; Walstra, Wouters, & Geurts, 2005). Furthermore, other minor compounds are also present, for instance proteose peptone, lactoferrin, lactoperoxidase and lysozyme (de Wit, 1998).

1.3 Whey protein composition

1.3.1 β-lactoglobulin

β-lactoglobulin is the dominant protein found in whey proteins which has a molecular weight of approximately 18.3 kDa. Its primary structure consists of 162 amino acid residues with one free thiol group (C121) and two disulphide bonds (C106-C119 and C66-C160) (Hoffmann & van Mil, 1999). The presence of a free thiol group in β-lactoglobulin plays an essential role in the thermal destabilization of whey proteins (Hoffmann, et al., 1999). The secondary and tertiary structure of β-lactoglobulin consist of 43-50% of β-sheet, 10-15% of α helix, and 15-20% of β-turn (Cayot & Lorient, 1997). The β-lactoglobulin conformation is pH-dependent; it exists as a monomer at acidic pH (<3) and as a dimer at pH between 5 and 8. (de Wit & Klarenbeek, 1984;

Kontopidis, Holt, & Sawyer, 2004; Vardhanabhuti & Foegeding, 2008). It associates to octamers at pH below 2 or above 8. At pH values above 9, reversible denaturation of β-lactoglobulin takes place (Cayot, et al., 1997). By increasing the temperature, the equilibrium will shift to monomers which is a prerequisite for heat induced aggregation (Hoffmann, et al., 1999). Since β-lactoglobulin is the major component of whey proteins, its properties tend to dominate the properties of whey proteins including the heat stability properties (Walstra, et al., 2005). Therefore, the denaturation temperature of whey proteins can be represented by the denaturation temperature of β-lactoglobulin. β-lactoglobulin is known to be very heat labile. In milk, it was found that β-lactoglobulin denatures much faster than α-lactalbumin (Vasbinder, van Mil, Bot, & de Kruif, 2001). Heating of β-lactoglobulin at 80^{o}C and neutral pH (6-7) for 20 minutes causes 80% of the protein to denature (Law & Leaver, 2000a). Furthermore, β-lactoglobulin can also interact with caseins through intermolecular disulphide bonds during milk heat treatment (Vasbinder & de Kruif, 2003), which leads to protein aggregation. β-lactoglobulin is pH-sensitive and has an IEP at approximately 4.0 to 5.2 (Bryant, et al., 1998; Kováčová, Synytsya, & Štětina, 2009). At pH-conditions around its isoelectric point (IEP), β-lactoglobulin produces aggregates with a size of 2000–2500 nm and has a very low solubility (Kováčová, et al., 2009).

1.3.2 α-lactalbumin

α-lactalbumin has a molecular weight of 14.2 kDa (Jambrak, Mason, Lelas, Paniwnyk, & Herceg, 2014). The IEP of α -lactalbumin is located at pH 4.8-5.1 (Bryant, et al., 1998). The secondary structure of α-lactalbumin consists of two main domains of the native molecule, namely α-helix (30%) and β-sheet (9%), which are connected by Ca^{2+}(Cayot, et al., 1997). Its structure exhibits a great flexibility and recovery of the native conformation due to its Ca^{2+} binding properties and the low degree of ordered secondary structure (Cayot, et al., 1997). Upon heating of pure α-lacatalbumin, cleavage of the disulphide bond in the α-lactalbumin structure occurs, resulting in the formation of dimers (Wijayanti, Bansal, Sharma, & Deeth, 2014). Despite having a lower denaturation temperature (62^{o}C), compared to β-lactoglobulin, α-lactalbumin is less heat labile (Bryant, et al., 1998; Law, et al., 2000a). The heat stability of α-lactalbumin is due to the absence of a free thiol group (de Wit, et al., 1984). Furthermore the presence of Ca^{2+} ions (Ca binding site) also contributes to the heat stability of α-

lactalbumin since the energy required to break the bonding is high. In fact, without the Ca^{2+} ions, the heat stability of α-lactalbumin is comparable to that of β-lactoglobulin (Haque, Aldred, Chen, Barrow, & Adhikari, 2013). The absence of a free thiol group and the presence of Ca^{2+} in its structure are responsible for the renaturation phenomenon observed during cooling of heated α-lactalbumin (Bernal & Jelen, 1985b; de la Fuente, Singh, & Hemar, 2002). α-lactalbumin is highly water soluble even at its isoelectric point due to the presence of a high amount of hydrophilic groups, which leads to its incapability to precipitate from milk at its isoelectric point (Swaisgood, 1982). α-lactalbumin has a good emulsifying activity which has been widely studied and reported (Lam & Nickerson, 2015; Suttiprasit, Krisdhasima, & McGuire, 1992; Zhai, et al., 2012).

1.3.3 Other minor proteins

Bovine serum albumin (BSA) of milk is physically and immunologically similar to blood serum albumin (Eigel, et al., 1984). It has 17 intramolecular disulphide bonds and one free sulfhydryl group (Eigel, et al., 1984). BSA has 582 amino acid residues with a molecular weight of about 66 kDa. The IEP of BSA is reported to be at pH 4.8-5.1 (Bryant, et al., 1998). BSA has a relatively low denaturation temperature of 64°C (Bryant, et al., 1998). Fatty acids were reported to provide stabilization of BSA against heat denaturation (de Wit, et al., 1984). BSA is a well-known whey protein for its gelling properties. During gelation of BSA, the amount of β-sheet, which is very low in the native form, increases while the amount of α-helices decreases. This transition is particularly critical in the gelation of BSA (Wijayanti, Bansal, & Deeth, 2014).

The other minor proteins present in whey proteins are immunoglobulins (IGg) which are glycoproteins with antibody properties (Cayot, et al., 1997). IgG, IgA and IgM are the main immunoglobulins in bovine milk and whey (de Wit, et al., 1984). The most heat sensitive immunoglobulin is IgM, whereas the most heat resistant type is IgG (McSweeney & Fox, 2013). Despite having a higher denaturation temperature compared to β-lactoglobulin and α-lactalbumin, the presence of BSA reduces the heat stability of immunoglobulins due to interaction of the free thiol group of BSA with immunoglobulins (Cayot, et al., 1997).

Other proteins such as lactoferrin, lysozyme, or lactoperoxidase are also present in a limited amount (de Wit, 1998). Lactoferrin, lactoperoxidase, and BSA have been reported to have anti-

cancer activities (Gill, et al., 2000). Furthermore, lactoferrin also exhibits antimicrobial activity (Farnaud & Evans, 2003). Lactoferrin had a relatively high IEP (± 8.0) (Ye & Singh, 2006).

For the sake of completeness, it can be mentioned that the milk fat globule membrane (MFGM) is also a promising source of dairy proteins. More information on this type of proteins can be found in the publications of Dewettinck, et al. (2008), Le, et al. (2011), Spitsberg (2005), and Lopez, et al. (2017).

1.4 Emulsifying activity of whey proteins

Whey proteins offer different functionalities which are useful for different applications in foods. However, in this study the focus is given especially on the role of whey proteins as food emulsifier in oil in water emulsions. Whey proteins have been known to offer good surface activity owing to their molecular flexibility and amphiphilic nature (Damodaran, 2005; Kato, et al., 1992). The most important functional property of whey proteins is their high solubility over a wide range of pH values, which is a principal prerequisite for their functional properties such as their emulsifying activity (Kinsella, Fox, & Rockland, 1986). Whey proteins have the ability to readily adsorb at the interface, reduce the interfacial tension at the oil and water interface, and also form an interfacial membrane around oil droplets preventing destabilization of the emulsion (Kinsella, Fox, & Rockland, 1986). Due to these abilities, whey proteins are considered as a good surfactant (Damodaran, 2005). Despite the excellent emulsifying properties, the heat lability of whey proteins limits the application of these proteins as industrial emulsifiers (Kato, et al., 1992).

Upon emulsification, whey proteins are adsorbed to the surface of the oil droplets followed by unfolding of the proteins. They unfold and rearrange so that the hydrophobic groups are anchored in the oil phase while the hydrophilic groups orient themselves in the aqueous phase (Euston, Finnigan, & Hirst, 2000). Once adsorbed, β-lactoglobulin forms a viscoelastic layer on the surface of oil droplets which is more viscous and rigid than that formed by low molecular weight surfactants and disordered proteins (such as caseins) (Lefèvre & Subirade, 2003). This viscoelastic layer can be formed through non-covalent interactions between adjacent adsorbed protein molecules, for instance by polymerization at the interface involving sulfhydryl-disulphide interchange reactions in the presence of amino acids containing sulfhydryl and disulphide group (Dickinson & Matsumura, 1991). This also causes the

adsorption of proteins to the surface of oil droplets to be irreversible. Furthermore, this layer can better adapt to shocks, compression, and distortion without rupture (Kinsella, et al., 1989). All of these aspects result in a stable emulsion.

Whey protein adsorption onto the oil droplet surface is selective and is influenced by pH, ionic strength, temperature, and protein concentration. Regarding the pH of the emulsion, β-lactoglobulin adsorbs more readily at alkaline pH (Yamauchi, Shimizu, & Kamiya, 1980). Furthermore, it works well in stabilizing emulsions especially at pH-conditions far from its isoelectric point, i.e. at a pH below 4 or above 6 (Demetriades, Coupland, & McClements, 1997a). Around its IEP, the emulsifying activity of WPI is poor since it has a low solubility.

During emulsification, in order to increase the adsorption of proteins to the surface of oil droplets, homogenization is normally used (Lefèvre, et al., 2003). The higher the surface area produced during homogenization, the more protein is adsorbed to the surface of the oil droplets (Lefèvre, et al., 2003). As long as the amount of whey proteins is sufficient to cover the newly produced surface area during homogenization, emulsions stabilized by whey proteins possess a great stability against coalescence (Singh, 2011). In the case of milk protein stabilized emulsions, these emulsions are prone to bridging flocculation at low protein concentration. On the other hand, when the concentration of protein is too high, depletion flocculation may take place. However, depletion flocculation has never been observed yet in whey protein stabilized emulsions (Singh, 2011).

The stability of whey protein stabilized emulsions is a result of different forces (repulsion and attraction) acting at the surface of oil droplets such as Van der Waals and electrostatic forces. When Van der Waals attraction between the emulsion droplets dominates, droplets tend to aggregate (Israelachvili, 1992). On the other hand, electrostatic forces are influenced by the charge of the droplets. Droplets with similar charges repel each other, resulting in a stable emulsion. At pH conditions near the IEP of the whey proteins, the net charge of the droplets is low and thus the repulsion between droplets is weak which in turn destabilizes the emulsions (Demetriades, et al., 1997a).

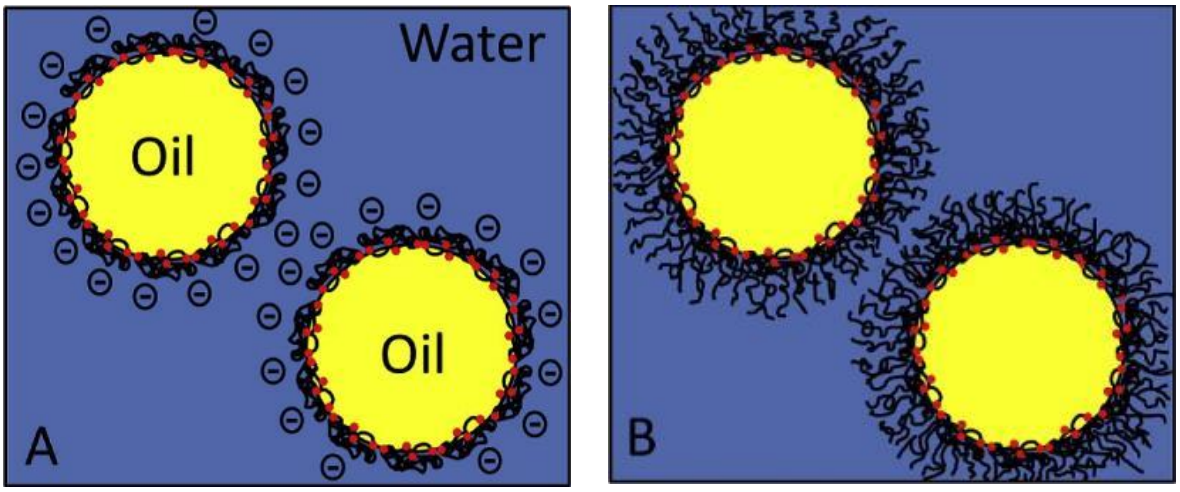


Figure 1.1. Emulsion stabilization in whey protein stabilized emulsions: (A) electrostatic repulsion and (B) steric stabilization. Red dots are hydrophobic parts of protein located at the oil phase (Lam & Nickerson, 2013).

In addition, the presence of electrolytes also influences the electrostatic repulsion between droplets. High ionic strength weakens the electrostatic repulsion between droplets due to the presence of counter-ions in the aqueous phase which shield the charges on the droplet surfaces (Demetriades, et al., 1997a). In whey protein stabilized emulsions, steric repulsion also plays an important role in stabilizing the oil droplets (Fig. 1.1) (Lam, et al., 2013). Besides pH and ionic strength, other factors influencing the stability of whey protein stabilized emulsions include the thermal history of the emulsions (Demetriades, et al., 1997b).

1.5 Heat treatment

In the food industry, different types of heat treatment such as pasteurization and sterilization are applied. These treatments are mostly applied to prolong the shelf-life of food products. Since whey proteins are very heat labile, the effect of heat treatment on whey proteins is very crucial. The influence of heat treatment will be discussed in two parts. The first part is dealing with the influence of heat treatment on whey proteins. It is then followed by the influence of heat treatment on emulsions stabilized by whey proteins.

1.5.1 Influence of heat treatment on whey proteins

Heat treatment causes changes in the conformation of whey proteins. When whey proteins are exposed to heat, changes in the size of the whey proteins are observed. This might occur due to the loss of cavity volumes which reduces the stability of the hydrophobic core of the globular structure (Dissanayake & Vasiljevic, 2009). Due to heat treatment, changes in hydrophobic, electrical, and structural properties which reduce the solubility and functionality of proteins are observed (Nakai & Li-Chan, 1985). Native whey proteins have a high solubility as a result of the large number of surface hydrophilic residues (Lee, et al., 1992). However, heat treatment of whey proteins leads to protein denaturation which in severe cases reduces the solubility of the whey proteins. A biopolymer should be highly soluble in the aqueous medium in order to provide an ideal steric stabilization (Dickinson & Galazka, 1991). Therefore, since the functionality of proteins is highly influenced by their solubility (Zayas, 1997b), it is important that whey proteins can maintain their solubility even after heat treatment.

The individual proteins present in whey proteins possess a different sensibility towards heat. Law, et al. (2000a) studied the heat stability of whey proteins and reported that for equivalent heating times, the heat instability of whey proteins is in the order of immunoglobulins > serum albumin/lactoferrin > β-lactoglobulin > α-lactalbumin. The denaturation temperature of β-lactoglobulin, α-lactalbumin, and bovine serum albumin is around 78, 62, and 64°C, respectively (Bryant, et al., 1998). Despite having a higher denaturation temperature than α-lactalbumin, β-lactoglobulin is more heat labile due to the presence of a free thiol group in its structure (Bryant, et al., 1998). α-lactalbumin does not undergo protein aggregation when it is heated alone. However, aggregates are formed when it is heated in the presence of β-lactoglobulin (Schokker, Singh, & Creamer, 2000). Since α-lactalbumin does not have any free thiol groups, it requires a free thiol group to aggregate which in this case is provided by β-lactoglobulin (Schokker, et al., 2000). Since thiol groups are also found in BSA, the presence of BSA has the same effect to the heat stability of -lactalbumin (McGuffey, Otter, van Zanten, & Foegeding, 2007; Schokker, et al., 2000). Whey proteins mainly consist of β-lactoglobulin. Thus, the thermal properties of whey proteins are greatly influenced by the thermal properties of β-lactoglobulin (Dissanayake, et al., 2009). Therefore it is reasonable to compare the behaviour of WPI during heating to that of β-lactoglobulin. The denaturation temperature of

β-lactoglubulin is also often used to represent the denaturation temperature of whey proteins (Law, et al., 2000a). Interestingly, β-lactoglobulin alone is more stable to heat than in whey protein isolate (Ryan, et al., 2012).

Heat treatment of whey proteins at temperatures below 60^{o}C induces reversible denaturation of the protein structure which involves a partial loss of the tertiary structure (de Wit, et al., 1984). When heated at temperatures above their denaturation temperature (>70^{o}C), whey proteins start to denature easily (Vasbinder, et al., 2001). This process is even more rapid in the presence of water (Haque, et al., 2013). On the other hand, in an environment with low water content, protein unfolding is probably reduced due to the low protein mobility (Gulzar, Bouhallab, Jeantet, Schuck, & Croguennec, 2011). Therefore the denaturation temperature of WPI in a powder form is higher than that in a solution (Gulzar, et al., 2011).

The conformation of β-lactoglobulin is mainly due to a combination of Van der Waals attraction forces, hydrophobic interaction, and electrostatic interaction (Demetriades, et al., 1997a; Harnsilawat, Pongsawatmanit, & McClements, 2006a; Pelegrine & Gasparetto, 2005). Initially, before heating, β-lactoglobulin exist as a dimer and monomer in which upon heating the equilibrium is shifted to the monomers (Nicolai, Britten, & Schmitt, 2011). When heating was performed at temperatures above the denaturation temperature of the β-lactoglobulin, it results in the disruption of their native structure (Kessler, et al., 1991). At this stage, the secondary and tertiary structure of β-lactoglobulin becomes unfolded, exposing the reactive amino side groups and the hydrophobic groups (-SH groups) which are normally buried (Anema & Li, 2003; Pelegrine, et al., 2005). This is also accompanied by the increased reactivity of the free thiol groups of whey proteins (Anema, et al., 2003). The exposed thiol groups in the newly formed monomers are able to form disulphide linkages by thiol/disulphide exchange reactions with other molecules (Hoffmann, et al., 1999). Due to this interaction, a small amount of oligomers (mainly dimers and trimers) is formed. Above a certain critical association concentration of oligomers, they associate and form protein aggregates (primary aggregates) (Nicolai, et al., 2011). This critical association concentration is highly influenced by the pH and ionic strength. The aggregates formed during heating are stable at lower protein concentrations, but associate into larger clusters or even a gel at higher concentrations (Nicolai, et al., 2011). The same author stated that the size of the aggregates gets bigger at

lower pH and higher ionic strength (Nicolai, et al., 2011). A schematic image of β-lactoglobulin aggregation can be found in Fig.1.2.

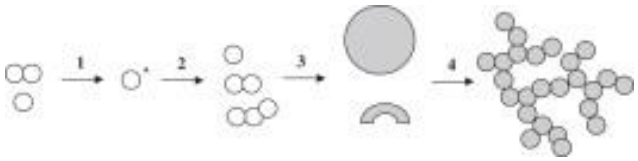


Figure 1.2. Schematic image of β-lactoglobulin aggregation obtained from Nicolai, et al. (2011): 1. Whey proteins exist as monomers and dimers, heating shifts the equilibrium towards monomers. 2. Formation of oligomers. 3. Formation of primary aggregates. 4. Formation of larger aggregates that can form a gel at certain concentrations.

In addition to the covalent disulphide bonds, noncovalent interactions, including hydrophobic, ionic and Van der Waals interactions, also play a role in the β-lactoglobulin aggregation process (Anema, et al., 2003; Hoffmann, et al., 1999; Pelegrine, et al., 2005). Their role in aggregation of β-lactoglobulin is more significant at higher temperatures (Vardhanabhuti, et al., 2008). The contribution of non-covalent bonding in aggregation and gelation processes is determined by the environmental conditions, such as pH, temperature and electrolyte concentration (Vardhanabhuti, et al., 2008).

To summarize, the processes involved in aggregate formation are (1) chemical aggregation through thiol groups-disulphide bonds exchange reactions, (2) physical aggregation of unfolded protein molecules or chemically formed aggregates, or (3) a complex combination of chemical and physical aggregation (Verheul, Roefs, & de Kruif, 1998). However the nature of cross-linking between proteins in the aggregates has not been known yet at a molecular level (Nicolai, et al., 2011). The authors suggested that it might involve hydrophobic, hydrogen bonding, and disulphide bonds. The latter is applicable at pH-conditions above the IEP of β-lactoglobulin (Nicolai, et al., 2011).

In milk, the behaviour of β-lactoglobulin during heating is also affected by the presence of caseins. It was found that the reaction order of β-lactoglobulin denaturation in skim milk was different from that in whey protein solution. The possible explanation was that there is influence of the caseins present in skim milk. The heated β-lactoglobulin is able interact with κ-casein at the periphery of the casein micelles through disulphide bonds leading to the

disruption of the casein micelles properties (Jang & Swaisgood, 1990; Vasbinder, et al., 2001). In the presence of caseins, β-lactoglobulin denatures to a greater extent (Kessler, et al., 1991). Whereas in whey protein solutions the order of reaction of β-lactoglobulin denaturation was 1.5, in skim milk and in the presence of caseins the reaction order of β-lactoglobulin denaturation was reported to be 2.0 (Kessler, et al., 1991).

For the sake of completeness, it has to be mentioned that heat treatment does not always have a negative impact as long as it is well controlled. For some cases, the changes that take place in whey proteins after heat treatment, such as partial denaturation, formation of soluble aggregates, or gelling of the proteins after heating, are in fact desirable. Soluble aggregates of whey proteins formed by controlled heating (90^{o}C for 10 minutes) have been found to have a better heat stability than native whey proteins which is attributed to the higher overall negative charge density, the smaller size, and the more compact structure (Ryan, et al., 2012). By controlling the denaturation rate, pH, and ionic strength, a gel can also be obtained which is beneficial to provide a desirable texture (Brncic, et al., 2009). A strong and elastic gel has been obtained by heating whey protein solutions at 85^{o}C and pH of 7.5 (Shimada & Cheftel, 1989). Adding of electrolytes, such as $CaCl_2$, enables the formation of a gel at a lower temperature (Barbut & Foegeding, 1993). Disulphide bonds due to sulfhydryl groups and disulphide bridge interchange reactions were mentioned to be responsible for the formation of the gel network obtained from the heated whey proteins (Shimada, et al., 1989). It is the same mechanism which is also responsible for the aggregation of whey proteins during heating.

1.5.2 Influence of heat treatment on whey proteins stabilized emulsions

The heat stability of whey protein stabilized emulsions is obviously influenced by the heat stability of the whey proteins. In whey protein stabilized emulsions, the droplet size of the emulsion increases as heat induced denaturation and aggregation take place, and reaches a maximum diameter. The higher the temperature, the sooner the maximum diameter is reached (Sliwinski, Roubos, Zoet, van Boekel, & Wouters, 2003). During heating of emulsions, three possible associations can occur, namely droplet-droplet interaction, protein-protein interaction, and protein-droplet interaction (Euston, et al., 2000). These authors stated that, upon heating, protein-droplet interaction was more favourable than droplet-droplet

interaction since the denatured non-adsorbed whey proteins have a higher hydrophobicity than the droplets surface (Euston, et al., 2000).

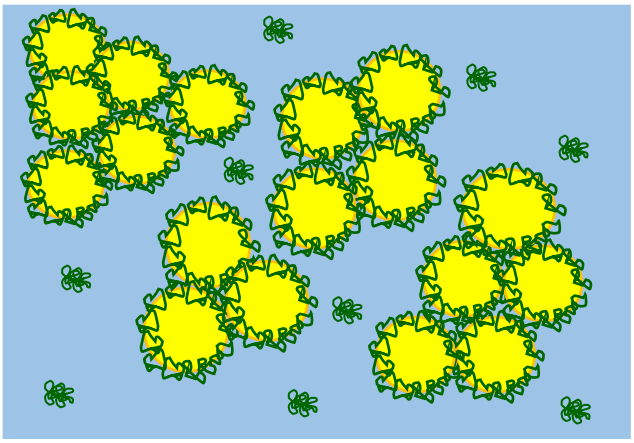


Figure 1.3 Schematic image of oil droplet aggregation due to the presence of denatured unadsorbed whey proteins which interact with adsorbed whey proteins. The unadsorbed whey proteins act as glue which binds the droplets together.

The rate of droplet flocculation in emulsions was reported to reach a maximum at temperatures around 65-80^{o}C and decrease upon further heating (Demetriades, et al., 1997b). A possible explanation is that at lower temperature the half unfolded proteins cannot effectively arrange their hydrophobic groups on the oil phase which increases the hydrophobicity of the surface of the oil droplets leading to flocculation. In contrast, at higher temperature the fully unfolded protein is able to arrange the hydrophobic groups to be fully attached on the oil droplet. Therefore, the surface of the oil droplet is less hydrophobic and the tendency of the oil droplets to aggregate is lower (Demetriades, et al., 1997b). Rearrangement of the aggregates takes place upon further heating which converts loose aggregates to smaller and more compact aggregates. This rearrangement can be explained by the increase of the number of contacts between proteins in the aggregates while at the same time the amount of available protein which has contact with the protein decreases (Sliwinski, et al., 2003). In emulsions, the amount of unadsorbed protein is important. Heating of oil in water emulsions results in the unfolding of non-adsorbed whey proteins exposing their hydrophobic groups which then induce association of the unadsorbed proteins with the adsorbed whey proteins on the surface of the oil droplets. These unfolded proteins act as a glue in between the oil droplets by associating with more than one droplet creating droplet flocculation and aggregation (Fig. 1.3) (Euston, et al., 2000). Preventing droplet aggregation in

heated emulsions is important. Droplet aggregation in heated emulsions increases the viscosity of these emulsion (Drapala, Auty, Mulvihill, & O'Mahony, 2016a) which alters the rheological properties of the products. In severe cases, a gel is formed upon heating of whey protein stabilized emulsions. For beverage production and in the dairy industry, this phenomenon is highly undesirable. Hereby, the concentration of whey proteins in the emulsion and the heating conditions are very important in determining the rheology and stability of the heated emulsion (Sliwinski, et al., 2003). Whey protein stabilized emulsions are considered to be heat stable if the emulsions can maintain their original consistency after heat treatment.

1.5.3 Factors influencing heat stability of whey proteins

Several factors have been identified to influence the thermal stability of whey proteins, for instance: temperature, heating time, pH, and electrolytes. The heat stability of whey protein stabilized emulsions is also influenced by these factors, since the thermal stability of whey protein stabilized emulsions is highly influenced by that of the whey proteins. Below, the effects of temperature, heating time, whey protein concentration, pH and electrolytes on the thermal stability of whey proteins are briefly discussed.

1.5.3.1 Temperature

Protein denaturation is highly influenced by temperature. The rate of protein denaturation is lower at lower temperature, and vice versa (Sliwinski, et al., 2003). Heating of emulsions at a medium temperature up to 60^{o}C results in a reversible denaturation (de Wit, et al., 1984). The denaturation temperature of whey proteins has been reported to be at temperatures of 75-80^{o}C (Bernal, et al., 1985b). Nevertheless, β-lactoglobulin has also been reported to start denaturing upon heating at 70^{o}C (Vasbinder, et al., 2001). At temperatures lower than 90^{o}C, protein denaturation is dominated by protein unfolding (Kessler, et al., 1991). Protein aggregation dominates the process once the temperature is increased to higher than 90^{o}C, (Kessler and Beyer 1991). According to Parris and Baginski (1991), heating of milk at 85^{o}C results in almost 95% denaturation of whey proteins. At 90^{o}C, approximately 100% of β-lactoglobulin is denatured (Vasbinder, et al., 2001).

1.5.3.2 Time

When protein stabilized emulsions are heated, the time required until visible aggregates are formed is called the heat coagulation time (HCT) (Dissanayake, et al., 2009). The denaturation rate increases as the heating time is prolonged and varies with pH (Law, et al., 2000a). At the same temperature, short heating leads to droplet aggregation resulting in loose aggregates. Further extension of the heat treatment will lead to deaggregation of the loose aggregates into smaller and compact aggregates (Sliwinski, et al., 2003). This phenomenon is specific for emulsions, since de-aggregation of aggregates upon further heating of β-lactoglobulin has not been reported (Sliwinski, et al., 2003).

1.5.3.3 Initial protein concentration

Aggregation occurs at lower temperature as the concentration of whey proteins is increased. The initial concentration of whey proteins is known to affect the hydrodynamic diameter of the aggregates (Purwanti, et al., 2011; Ryan, et al., 2012; Sliwinski, et al., 2003). At relatively high whey protein concentration (9%), protein unfolding was reported to be highly limited (Ryan, et al., 2012; Zhu & Damodaran, 1994). The rate of aggregation in emulsions is also highly influenced by the protein content of the emulsion: the higher the protein content, the higher the aggregation rate (Euston, et al., 2000). Sliwinski, et al. (2003) found that heating emulsions at temperatures ranging from 55 to 95°C containing 1.5% of WPI did not have a noticeable effect on the average volume-surface diameter ($d_{3,2}$) of the emulsions. On the other hand, when the WPI concentration was increased to 3%, heat treatment showed to have an impact on the average droplet size. The authors found that this phenomenon was due to the effect of non-adsorbed proteins in the aqueous phase; the amount of non-adsorbed proteins in emulsions containing 3% of WPI was 4 times higher than in emulsions containing 1.5% protein. Since the amount of non-adsorbed protein is essential for protein aggregation, removing the non-adsorbed protein from the aqueous phase of an emulsion will reduce the aggregation rate of the emulsion regardless of the initial concentration of protein in the emulsion (Euston, et al., 2000).

1.5.3.4 pH and electrolytes

The pH influences the charge density of whey proteins, which can reduce the intramolecular electrostatic repulsion and promote protein unfolding allowing protein aggregation (Law, et al., 2000a). In general, the denaturation temperature of β-lactoglobulin decreases as the pH is decreased from 9.0 to 3.0 (Damodaran & Paraf, 1997). At pH values below 3.0, heat denaturation of β-lactoglobulin seemed to be reversible as long as the heating temperature did not exceed 120^{o}C and when performed using a short heating time. On the other hand, irreversible denaturation was observed when the same treatment was applied to β-lactoglobulin at pH conditions above 3.5 (Damodaran, et al., 1997). Harwalkar (1980) found that at pH 2.5, heating caused the unfolding of protein which was not accompanied by protein aggregation. At very acidic pH (± 2.0), disulphide exchange, which is responsible for heat-induced protein aggregation at high pH, is prevented (Kavanagh, Clark, & Ross-Murphy, 2000). Pelegrine, et al. (2005) reported that the IEP point of WPI was at 4.5, while β-lactoglobulin alone was at pH 5.2. At a pH near the isoelectric point of the protein, the inter- as well as intra-molecular repulsive forces are very low, leading to protein aggregation (Ju & Kilara, 1998). Hence, the heat-induced aggregation rate of β-lactoglobulin is high in the pH range of 5.2 to 6.1. At pH-values higher than 6.1 to 6.8, the rate of aggregation decreases. However, in this pH range, when the heating time was prolonged, the rate of denaturation increased. Whey proteins were also reported to possess a low heat stability at pH 6.8 and 7.0, especially in the presence of NaCl (Demetriades, et al., 1997b; Ryan, et al., 2012). At pH 7, β-lactoglobulin seems to be unstable since at this pH, ionization and thiol group activity increased to the point that it can cause globular proteins to unfold and enhance denaturation (Law & Leaver, 2000b). Whey protein stabilized emulsions have also been reported to possess a low heat stability at pH 6.8 and 7, especially in the presence of electrolytes (Demetriades, et al., 1997b). At pH 7, it was observed that disulphide bonds, which are responsible for droplet flocculation, are formed more rapidly during heating than at lower pH (±3) (Kim, Decker, & McClements, 2004). The rate of denaturation was found to increase significantly as the pH was increased to 8.8 (Law and Leaver 2000).

As for electrolytes, the aggregation temperature of whey proteins shifts to a lower temperature at higher electrolyte concentrations (Fitzsimons, et al., 2007). Adding $CaCl_2$ before heat treatment was found to significantly increase the denaturation rate of all whey

proteins except for immunoglobins (Law, et al., 2000a). Adding electrolytes to protein solutions causes a reduction of the intermolecular repulsion (Vardhanabhuti, et al., 2008). These authors conducted a research by mixing β-lactoglobulin, α-lactalbumin, and dextran sulphate with NaCl and distilled water to give various protein concentrations. At 60 mM of NaCl, an increase in the turbidity, radius, and molecular mass of the samples was observed. Ryan et al. (2012) measured the turbidity of heat-treated native WPI (90^{o}C, for 5 minutes) and β-lactoglobulin in the presence of NaCl (0-108 mM) using spectrophotometry at 400 and 600 nm. The authors found that heat-treated native WPI and β-lactoglobulin had a low turbidity and remained highly soluble even in the presence of 54 mM NaCl. A higher turbidity and lower protein solubility were obtained at higher concentrations of NaCl (Ryan, et al., 2012).

In an emulsion stabilized by whey proteins, adding a high concentration of NaCl into the aqueous phase of an emulsion increases the ionic strength of the aqueous phase and eventually causes destabilization of the emulsion in a wide range of pH conditions (Demetriades, et al., 1997a). A high ionic strength of the aqueous phase will decrease the electrostatic repulsion between droplets, and thus enhance the tendency of aggregation and association of the oil droplets. The presence of ions in the aqueous phase will counteract the charges on the surface of the oil droplets, leading to a reduction of the repulsive force (Demetriades, et al., 1997b). These are all leading to further destabilization of the emulsion during heat treatment. Upon heating, increasing the salt concentration of the emulsion from 25-100 mM increased the size of the particles due to droplet flocculation and reduced the temperature at which the maximum size was reached (Demetriades, et al., 1997b). Droplet flocculation caused the viscosity of the emulsions to increase and exhibit a shear thinning behaviour. In addition, increasing the concentration of salt broadens the range of temperatures in which the emulsions exhibit an increased viscosity and shear thinning behaviour (Demetriades, et al., 1997b).

1.6 Improving heat stability of whey proteins

Whey proteins have been used in many food applications. However, the drawback of using whey proteins on industrial scale is their thermal instability (Ryan, et al., 2012). The solubility of proteins is an important factor for their application and functionality (Zayas, 1997b). Since heat treatment is almost unavoidable in food processing, it is of utmost importance to

improve the heat stability of whey proteins (Nakai, et al., 1985). This way, the quality of the food products, i.e. particle size and consistency, can also be maintained even after severe heat treatment, such as sterilization. In the dairy industry, improvement of the heat stability of whey proteins also offers a great advantage as it can reduce the formation of a protein deposit on the walls of heat exchangers. Furthermore, improving the heat stability of whey proteins will eventually improve the heat stability of whey protein stabilized emulsions. In order to obtain heat stable whey proteins with improved functional properties, modification of the molecular structure of the whey proteins can be performed (Demetriades, et al., 1997a). Steric, charge, and hydrophobic properties are important to maintain the solubility of heated proteins (Nakai, et al., 1985). These factors can be taken into account for selecting a method to be used for modifying the structure of whey proteins. Several methods to modify the structure of proteins in order to improve their heat stability have been proposed including physical modification, enzymatic modification, and chemical modification. Physical modification of proteins can be performed by partial denaturation of proteins or protein unfolding to increase the hydrophobicity of the proteins. This can be performed by exposing the proteins either to heat or to a hydrostatic pressure under controlled heating and shear conditions. However, this method does not completely solve the problems since the partially denatured proteins are still sensitive to heating and pH. Furthermore, the process of the partial denaturation itself is difficult to be controlled (Damodaran, 2005). Another alternative is enzymatic modification of proteins via hydrolysis and polymerization. Protein hydrolysis can be performed using pepsin and trypsin, while polymerization of proteins is possible with transglutaminase (Damodaran, 2005). The latter has been applied for improving the thermal stability of whey proteins through formation of covalent cross-links between reactive proteins (Truong, Clare, Catignani, & Swaisgood, 2004; Wang, Zhong, & Hu, 2012; Zhong, Wang, Hu, & Ikeda, 2013). Chemical modification of proteins is performed by changing either the structure of the proteins at the secondary, tertiary and quaternary levels, or the hydrophobic to hydrophilic ratio (Damodaran, 2005). Acylation, phosphorylation, alkylation, sulfitolysis and the amino-carbonyl reaction are some available methods to chemically modify proteins. Nevertheless, based on nutritional and safety considerations, among these methods, phosphorylation and amino-carbonyl (Maillard reaction) methods are more advisable for application in food products (Damodaran, 2005). In contrast to other chemical methods, the Maillard reaction is a spontaneous and naturally occurring reaction. This process only requires

the addition of reducing sugars or polysaccharides and no additional chemical is needed. Hence, these products can be safely incorporated into food systems without using undesirable chemical catalysis (Oliver, Melton, & Stanley, 2006). Recently, combining proteins with other biopolymers, such as pectin, to improve the functionality of the proteins has become popular.

Besides structure modification, the heat stability of proteins can also be improved by adding compounds which can prevent protein aggregation such as dihydrolipoic acid (DHLA) or N-ethylmaleimide (NEM). These compounds improve the thermal stability of β-lactoglobulin by blocking the reactive thiol groups preventing protein aggregation upon heating (Wijayanti, Bansal et al. 2014). However, some of these thiol blocking compounds are not food grade.

Among the available methods mentioned above, combining whey proteins with other biopolymers such as polysaccharides seems to be a promising method to improve the heat stability of whey proteins. Biopolymers such as pectin, gum Arabic, and carrageenan are all food grade and have been widely used in food applications. As polysaccharides are mostly hydrophilic, the presence of the hydrophilic groups from the polysaccharides might enhance the hydrophilicity of the proteins. Furthermore, due to the growing interest in natural emulsifiers, the application of this method would enable the production of emulsion without synthetic surfactant, which are sometimes referred to as so-called “clean-labeled emulsions” (Garti, 1999a; Ozturk & McClements, 2016).

1.7 Interaction of whey proteins and polysaccharides

Whey proteins can be combined with other components, such as emulsifiers or thickeners to improve their functionality (Demetriades, et al., 1997a). Food products are composed of a wide range of ingredients such as proteins and carbohydrate-based polysaccharides (Ye, 2008). Some polysaccharides are known to have a good emulsion stabilizing activity (Dickinson, 2009). Whereas proteins adsorb at the oil-water interface during emulsification to form a viscoelastic layer, polysaccharides offer colloid stability through their thickening and gelation behaviour in the aqueous phase. Combining the hydrophobic properties of proteins and the hydrophilic properties of polysaccharides has been reported to improve the functionality and heat stability of whey protein (Dickinson, 1993a). However, mixtures of biopolymers are often unstable, which leads to separation of the mixture into two phases (Ye,

2008). Thus, certain techniques to combine proteins and polysaccharides are needed in order to achieve the desirable properties and stable biopolymer complexes. Combining whey proteins and polysaccharides can be performed via either electrostatic interaction or conjugation by covalently linking the polysaccharides to whey proteins.

1.7.1 Electrostatic interactions of whey proteins and polysaccharides

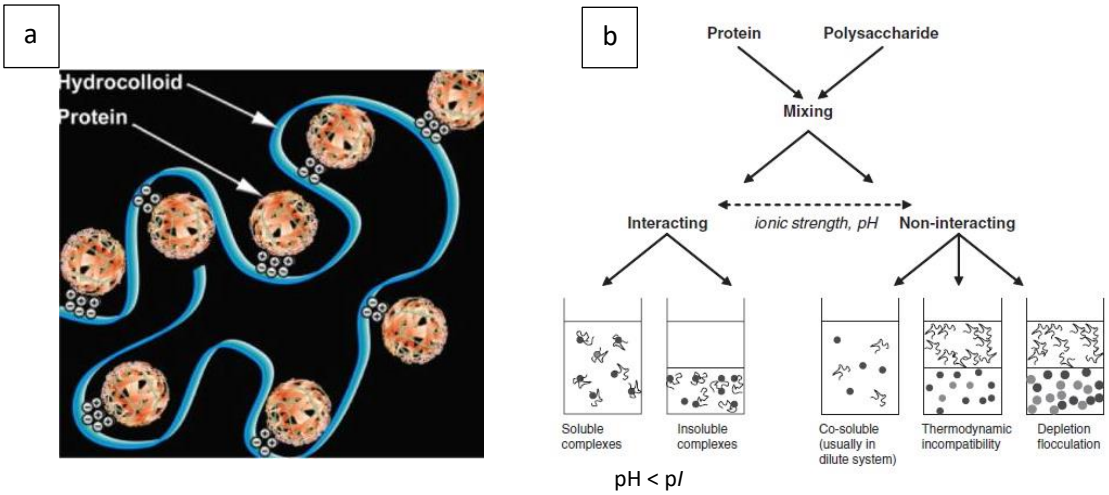


Figure 1.4 Schematic image of whey protein-gum Arabic electrostatic complex (Klein, Aserin, Ishai, & Garti, 2010) (a) and protein –polysaccharide complex formation via electrostatic interaction (Goh, Sarkar, & Singh, 2009).

Electrostatic interaction between proteins and polysaccharides is highly influenced by pH. Figure 1.4 shows the schematic image of whey protein-gum Arabic and protein–polysaccharide complex formation via electrostatic interaction (Goh, et al., 2009; Klein, et al., 2010). Strong electrostatic interaction between proteins and polysaccharides is possible when the biopolymers are oppositely charged. In the case of proteins and anionic polysaccharides, at a pH below the IEP of the protein, proteins are positively charged while anionic polysaccharides are negatively charged. At this condition, proteins and polysaccharides are strongly attracted to each other. When the amount of charges is not equal between the two biopolymers, soluble complexes are formed. On the other hand, when the net charge of the complexes is close to zero, the complexes tend to aggregate, thus forming insoluble complexes (Goh, et al., 2009). At a pH around the IEP of the whey proteins, electrostatic interaction between proteins and polysaccharides is possible and protects whey proteins from

pH induced aggregation (Jones, Decker, & McClements, 2009). At a pH below the IEP of the protein, the positively charged protein interacts with the negatively charged polysaccharides. The interaction takes place between the NH_3^+ groups of the protein and the negatively charged COO^- groups of the polysaccharide. The same phenomenon takes place when whey proteins are mixed with anionic polysaccharides at a pH above the IEP of whey proteins. At neutral pH (±7) (pH>IEP), electrostatic interaction is less likely to occur in mixtures of proteins and polysaccharides. Thermodynamic incompatibility is likely to take place at this pH (Goh, et al., 2009). In addition, phase separation caused by depletion flocculation can also take place which is prevalent at a high concentration of polysaccharides. Harnsilawat, et al. (2006a) reported that complexation of β-lactoglobulin and sodium alginate did not take place at pH 6 and 7 due to electrostatic repulsion between the biopolymers. However, Einhorn-Stoll, Glasenapp, and Kunzek (1996) stated that a weak electrostatic interaction between protein and pectin is still possible at pH 7 (Fig. 1.4). The negatively charged pectin can interact with the positively charged patches of protein resulting in a weak electrostatic interaction. The authors further reported that hydrophobic association of the ester group of polysaccharides and the non-polar side chain of the protein and hydrogen interaction between the OH and NH_2 group might also take place at pH 7. These interactions are weaker than the electrostatic interaction; however, they are responsible for the stabilizing effect of a pectin and protein mixture in emulsions (Einhorn-Stoll, et al., 1996).

The methods used for electrostatic complexation are relatively simple. Improvement of the functionality and thermal stability of whey proteins through electrostatic interaction with pectin has been previously reported (Jones, et al., 2009). However, the electrostatic complexation is highly affected by the environmental conditions such as pH, ionic strength, and temperature (Schmitt, Sanchez, Desobry-Banon, & Hardy, 1998). The presence of electrolytes and change of pH might break or weaken the complexation between the biopolymers. Therefore, electrostatic complexes have a very narrow range of optimum pH and are highly sensitive to the pH, which limits their application (Dickinson, 1993a). Heating is sometimes required to stabilize the electrostatic complexes at neutral pH (Gentès, St-Gelais, & Turgeon, 2010).

1.7.2 Conjugation of whey proteins and polysaccharides

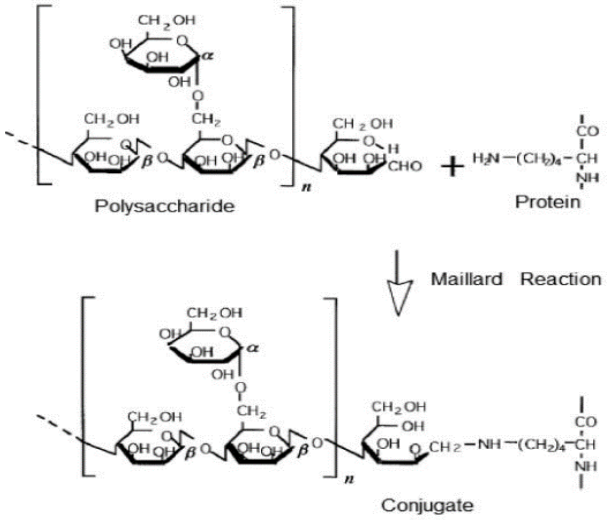


Figure 1.5. Schematic image of protein and polysaccharide conjugation via a Maillard type reaction (de Oliveira, Coimbra, de Oliveira, Zuñiga, & Rojas, 2016).

Whey proteins and pectin can be conjugated by applying heat to a mixture of protein and polysaccharide. In this method, the protein and the polysaccharide are heated at slightly elevated temperatures for a certain period of time to induce Maillard type reactions. This will result in a glycoprotein, a compound in which the polysaccharide is covalently linked to the protein (Dickinson, 2008). No additional chemical is needed in this method making this method safe for food applications (Damodaran, 2005; Kato, 2002).

Conjugation of proteins and polysaccharides can be performed either in the wet state or dry state. In the wet state, the protein and polysaccharide mixture is heated in solution. This method has been successfully used to improve the functionality of gelatin and whey proteins (Diftis, Pirzas, & Kiosseoglou, 2005; Zhu, Damodaran, & Lucey, 2010). The drawback of this method is that there is a risk of contamination due to microbial growth (Liu & Zhong, 2013). Furthermore, handling and storage of the liquid product are more challenging than that of dry products(Liu, et al., 2013). It was also suggested that the products obtained from dry heating have a better long term stability than that obtained from wet heating (Liu, et al., 2013). Regarding the impact of conjugation to the structure of the protein, conjugation via wet state heat treatment has been found to cause important structural changes resulting in a specific denatured β-lactoglobulin monomer, which is covalently associated via the free thiol group (Morgan, Léonil, Mollé, & Bouhallab, 1999). On the other hand, this phenomenon is restricted

in dry state heat treatment. Xia, et al. (2015) found that upon dry heating of BSA and dextran, the attachment of dextran to BSA was able to minimize the loss of its secondary and tertiary structure during dry heat treatment and protect BSA from aggregation. Hence, conjugation of proteins and polysaccharides in dry state is more preferable due to the advantages that the method offers.

Dry heat treatment is performed by heating a dry mixture of proteins and polysaccharides. Upon dry heat treatment, Maillard reactions take place resulting in a covalently linked protein and polysaccharide (Fig. 1.5). To be specific, the conjugation of whey proteins and polysaccharides via dry heat treatment is basically based on the Amadori arrangement which takes place at the early stages of the Maillard reaction (Oliver, et al., 2006). In the Maillard reaction, the amino groups of a protein interact with the free carbonyl groups of polysaccharides. The amino groups that actively participate in the Maillard reaction are mostly ε-amino groups of lysine. However, other groups such as imidazole groups of histidine, indole groups of tryptophan, and guanidine groups of arginine are able to participate in the Maillard reaction to a lesser extent (Ames, 1998).

In general, the Maillard reaction is composed of three different stages, namely early, intermediate/advanced, and final stage (Jimenez-Castano, Villamiel, & López-Fandiño, 2007; Liu, Ru, & Ding, 2012; Oliver, et al., 2006). At the early stages of the Maillard reaction, carbonyl groups condense with free amino groups to form a Schiff base accompanied by water release. The Schiff base then cyclizes to the corresponding N-glycosylamine followed by the irreversible Amadori rearrangement resulting in 1-amino-1-deoxy-2-ketose (Amadori product). However, if the Maillard reaction is performed using ketoses containing sugar, the reaction will follow the Heyn's rearrangement resulting in 2-amino-2-deoxyaldose (Heyn's product) (Oliver, et al., 2006). These intermediate products obtained might undergo further polymerization into brown colour at the advanced and final stages of the Maillard reaction (Lertittikul, Benjakul, & Tanaka, 2007). The same authors reported that formation of these intermediate products was higher when the initial pH of the sample before heat treatment was more alkaline.

Following the early stage, the intermediate stage of Maillard takes place. At this stage, the Amadori/Heyn's products are transformed via different pathways depending on the pH of the

environment (Oliver, et al., 2006). The products formed in the degradation of Amadori/Heyn's products are highly reactive and able to involve in various transformation reactions such as oxidation, cyclization, hydrolysis, fragmentation, and free radical reaction. Colour development has also been detected at the intermediate stage. Nevertheless, a more intensive colour development takes place at the final stage of the Maillard reaction with melanoidins as the final products (Jimenez-Castano, et al., 2007; Liu, et al., 2012; Oliver, et al., 2006). Nevertheless, in the case of protein and polysaccharide conjugation, the Maillard reaction should be well controlled to avoid the advanced stage of Maillard reaction. The latter has been found to produce protein-polysaccharide conjugates with low solubility due to cross linking and polymerization, and thus exhibits poor functionality (Kato, 2002; Oliver, et al., 2006). For food applications, conjugates with low solubility and intense brown colour are undesirable (Oliver, et al., 2006). Furthermore, since the production of flavours at the early stages of the Maillard reaction is highly limited (Oliver, et al., 2006), off flavour formation in the food products can be avoided. The product of dry heat treatment via Maillard reaction is a hybrid compound with high molecular weight (Lertittikul, et al., 2007; Setiowati, Vermeir, Martins, De Meulenaer, & Van der Meeren, 2016). This new compound (conjugate) seems to be responsible for the improved functionality and thermal stability of the conjugate.

Glycation of proteins, as well as conjugation of proteins and polysaccharides via the Maillard reaction influences the functional properties of proteins by changing their charge, solvation, and conformation (Nakamura, Kobayashi, & Kato, 1994). The functional properties of the protein-polysaccharide conjugates are remarkably different from the original biopolymers (Akhtar & Dickinson, 2007). The excellent emulsion stability offered by protein-polysaccharide conjugates is due to the fact that the biopolymers work synergistically to stabilize the emulsion. Upon emulsification, the protein moieties of the conjugates are adsorbed on the oil-water interface (Kato, 2002) and thus polysaccharides are carried by the protein and become available on the surface of the oil droplets. The latter is not possible or very limited when it is not conjugated to the protein. Upon adsorption, the hydrophobic groups of the protein are anchored in the oil droplets while the hydrophilic groups of the polysaccharides orient themselves in the aqueous phase (Shu, Sahara, Nakamura, & Kato, 1996b). Protein-polysaccharide conjugates stabilize the emulsion through a steric hindrance effect which inhibits coalescence of oil droplets during emulsification (Shu, et al., 1996b) resulting in a

smaller droplet size. Polysaccharides provide a protective viscoelastic layer which enhances the stability of the emulsion over time (Kato, 2002). Furthermore, the presence of additional hydrophilic groups from the polysaccharides increases the hydrophilicity of the oil droplets in the emulsion, which also enhances the stability of the emulsions (Akhtar, et al., 2007; Diftis & Kiosseoglou, 2006b; Kinsella, et al., 1989). In addition to the steric stabilization, the charge of the conjugates also plays an important role in stabilizing the conjugates. Thus, the stabilizing activity of protein-polysaccharide conjugates is a combination of steric and electrostatic stabilization.

Besides improving the emulsifying properties of proteins, conjugation of proteins and polysaccharides has also been reported to improve the heat stability of proteins (Aoki, et al., 1999; Jiménez-Castaño, López-Fandiño, Olano, & Villamiel, 2005). Glycosylation of proteins was suggested to increase their denaturation temperature; nevertheless, the rise of denaturation temperature might not be the only factor that is responsible for the limited aggregation of proteins upon heating (Liu, et al., 2013). Liu, et al. (2013) stated that electrostatic repulsion is not the main cause of the improved heat stability of glycated WPI. By comparing conjugates made with proteins and polysaccharides of different molecular weight, the authors found that the chain length of the polysaccharides had a bigger influence on the heat stability of the conjugates. Polysaccharides with longer chain length exhibited a greater steric repulsion leading to a better heat stability. It seems that the presence of polysaccharides in the protein-polysaccharide conjugates stabilizes the protein against heat by preventing the unfolded protein to aggregate through its steric stabilization activity (Schmitt, et al., 1998; Shu, et al., 1996b). Moreover, protein-polysaccharide complexes also exhibit an excellent heat stability at a pH around the IEP of the protein in which conditions normally without conjugation the protein is highly heat labile (Jiménez-Castaño, López-Fandiño, et al., 2005).

Compared to protein-polysaccharide complexes obtained from electrostatic interaction, protein-polysaccharide conjugates obtained from dry heat treatment are superior. The complex formation of proteins with polysaccharides via Maillard type reactions is more stable at a wider range of pH values and various ionic strengths without precipitation as compared to that obtained from electrostatic interaction (Dickinson, 2003). Furthermore, conjugation of proteins and polysaccharides reduces the sensitivity of the protein to pH due to alteration of

the net charge on the protein molecule which causes changes in the nature and magnitude of the intermolecular forces between protein molecules (Kinsella, et al., 1989; Kuntz & Kauzmann, 1974). Upon conjugation, the overall net charge of the conjugates will be shifted to more negative values since there is a reduction of the number of lysine residues which is the contributor of positive charges (Liu, et al., 2013).

The question may arise to what extent the Maillard reaction might produce unsafe and toxic products. Some products of the Maillard reaction have been reported to be mutagenic or carcinogenic (de Oliveira, et al., 2016). However, most of these compounds are produced at the final stage of the Maillard reaction while during formation of the conjugates, the advanced stage of the Maillard reaction is avoided. Hence, it is expected that those compounds are not formed. Jing and Nakamura (2005) conducted research to test the toxicity and mutagenicity of glycosylated products in rats (protein-polysaccharides conjugates). They found that the conjugates were safe and no toxic compounds were found in the conjugates. Hence, the product of glycosylation prepared in well-controlled conditions is suitable for food application (Jing, et al., 2005; Sanmartín, Arboleya, Villamiel, & Moreno, 2009). Furthermore, it is worth mentioning that protein and polysaccharide glycosylation has been used to reduce the allergenicity of some proteins and has shown positive results (Nakamura, et al., 2008; Usui, et al., 2004).

1.8 Influencing factors

In order to achieve protein-polysaccharide conjugates with optimum functionality, it is important to control the heat treatment. A good understanding of the key reaction parameters influencing the glycation and side reactions as well as their influence on the protein functionality is of utmost importance. Conjugation of proteins and polysaccharides is influenced by several factors, such as temperature, time, pH, relative humidity, as well as type and concentration of protein and polysaccharide (Ames, 1998).

1.8.2 Combination of time, temperature, and relative humidity (RH) / water activity (a_w)

The dry heating method involves lyophilisation of a solution of a protein and a reducing sugar or oligo/polysaccharide, which is followed by equilibration and incubation at the desired a_w or RH under controlled temperature for a certain period of time (Kato, 2002). It was reported

that conjugation of β-lactoglobulin and dextran at higher temperature, lower water activity (a_w) and lower ratio of β-lactoglobulin to dextran resulted in a higher formation rate of Amadori compounds which correlated with a higher degree of conjugation (Jiménez-Castaño, Villamiel, Martín-Álvarez, Olano, & López-Fandiño, 2005). Martinez-Alvarenga, et al. (2014a) reported that increasing the incubation temperature from 50 to 60^{o}C enabled the increase of the degree of conjugation from 26 to 40%. Besides an increase of the reactivity of the carbonyl group and amino group by incubation at higher temperature, the authors also suggested that this could be due to the greater unfolding that occurred to the protein structure which consequently exposed a higher amount of reactive functional groups, such as lysine.

Regarding the heating time or incubation time, the duration of the incubation should be well controlled and as short as possible to prevent the reaction reaching the advanced stage and to limit the polymerization and browning (Aoki, et al., 1999; Liu, et al., 2013). Furthermore, Xia, et al. (2015) have reported that upon conjugation of BSA and dextran, a longer incubation time decreased the rate of the dextran attachment to WPI, which might be due to the steric hindrance of the dextran which was already attached to the WPI. The optimum time for conjugate formation might differ between each process depending on the type of polysaccharide and temperature; it can range from hours to weeks.

The optimum a_w for the Maillard reaction is located between 0.5 and 0.8 (Buera & Karel, 1993; Oliver, et al., 2006). However, a wider range of optimum a_w values for Maillard was reported by Martinez-Alvarenga, et al. (2014a): in their study, the optimum a_w was observed to be in between 0.3 and 0.8. At higher a_w the reactants are too diluted thus the conjugation rate is lower. On the other hand, at a very low a_w the mobility of the reactant is too restricted (Buera, et al., 1993; Martinez-Alvarenga, et al., 2014a) and as a consequence the conjugation is limited. Conjugation of proteins and polysaccharides was also reported to be optimal when it was performed at a relative humidity of 65% and 79% (Kato, 2002).

1.8.3 Type of polysaccharide

The type of polysaccharide highly influences the functionality and heat stability of the conjugates since steric stabilization was reported to play an important role in the functionality and heat stability of the conjugates. The functionality of proteins decreases when a high

amount of carbonyl groups is attached to the amine groups of the proteins (Kato, 2002). Furthermore, the advanced stage of the Maillard reaction has been found to highly reduce the solubility of the conjugate (Kato, 2002). This is undesirable since the functionality of proteins is largely affected by their solubility. This phenomenon has a higher possibility to take place when the conjugation is performed with monosaccharides as well as oligosaccharides (Kato, 2002). In addition, when short chain polysaccharides or polysaccharides rich in reducing sugar are used, the reaction should also be strictly controlled to prevent the advanced stage of the Maillard reaction in which polymerization and browning dominate (Aoki, et al., 1999). Polysaccharides are less reactive than monosaccharides, and thus the advanced and final stage of the Maillard reaction can be more easily prevented. It was found that the brown colour development and protein polymerization during Maillard reaction was suppressed when conjugation was performed with long chain polysaccharides (Aoki, et al., 1999). Furthermore, the longer the chain of the polysaccharides, the better the emulsifying activity of the conjugates will be (Shu, Sahara, Nakamura, & Kato, 1996a). Hence, conjugation of proteins with polysaccharides is found to be more interesting for industrial application (Kato, 2002). As far as the molecular weight is concerned, it was reported that whey proteins conjugated with a lower molecular weight of dextran underwent more structural changes than those with a higher molecular weight of dextran (Spotti, et al., 2014). Kato (2002) found that saccharides with a molecular weight of at least 10 kDa are suitable to be conjugated with proteins as they are able to increase the functionality of the proteins as emulsifier.

Long chain and high molecular weight polysaccharides that can be utilized include pectins, gums, dextrans, and galactomannans. In this study, pectin was chosen due to its abundant availability in nature and its functional properties. Furthermore, whey proteins have been reported to be thermodynamically compatible with pectin which is important to obtain optimal conjugation (Einhorn-Stoll, Ulbrich, Sever, & Kunzek, 2005). Due to its compatibility, the whey proteins and pectin will be in close contact upon mixing and form a homogeneous mixture of the biopolymers, thus promoting better conjugation between whey proteins and pectin (Einhorn-Stoll, et al., 2005).

1.9 Pectin

Food emulsions such as milk, salad dressings, desserts, and beverages are thermodynamically unstable and susceptible to destabilization phenomena, such as gravitational separation or creaming, flocculation and coalescence (Guo, et al., 2014). Hydrocolloids have been widely used to stabilize these types of products. Pectin is one of the most widely used hydrocolloids as a stabilizing agent of emulsions like oil in water emulsions to prevent or slow down the creaming of the emulsion by modifying the rheological properties of the continuous phase (Dickinson, 2009). Pectins have a long chain and high molecular weight which makes them suitable to be conjugated with whey proteins.

Pectin is a polymer naturally occurring in plant cell walls especially in young tissues of fruits and vegetables, which acts as a cementing agent between cells and ensures the consistency of the plant tissues. The composition of pectin is influenced by the raw material, location, environmental factors and the method used for its extraction (Lozano, 2006). Pectin is an anionic polysaccharide composed of mainly galacturonic acid covalently linked with α-1,4-glycosidic bonds and polymerized with carboxylic acid groups as the main constituent backbone with side chains of galactose, xylose, and arabinose which gives acidic properties to pectin (Fig 1.6) (Walter, 1991). The carboxyl groups along with the pectin chain can be esterified with methanol resulting in methoxyl groups. Based on the degree of esterification, pectins are grouped into two main groups namely high-methoxyl pectin (HMP) with a degree of esterification of more than 50% and low-methoxyl pectin (LMP) with a degree of methylation less than 50% (Thakur, Singh, Handa, & Rao, 1997).

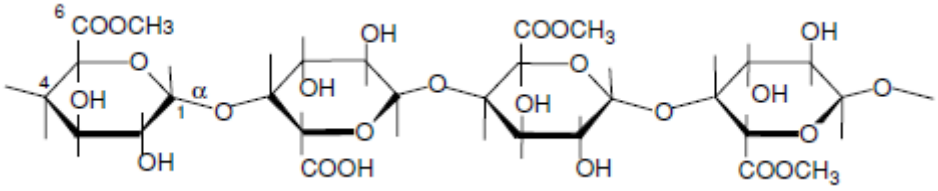


Figure 1.6 Pectin with covalently linked α-1,4- D-galacturonic acid (GalA) units (Chan et al., 2016).

High methoxyl pectin has a high amount of esterified groups along the galacturonic acid chains. It forms gels at low pH and in the presence of sugars. A decrease in pH and increase in sugar concentration enhance the HMP gel strength and the setting temperature (Chan, Choo,

Young, & Loh, 2016). HMP requires a minimum amount of soluble solids in order to form a gel (Walter, 1991). LMP, on the other hand, is obtained by reducing the number of methoxyl groups by a controlled de-esterification of high methoxyl pectin. LMP is highly sensitive to calcium gelation and less sensitive to acidity. The presence of calcium ions increases the gelling behaviour and the viscosity of LMP (Chan, et al., 2016). LMP is able to hydrate and form gels in cold or hot water. Furthermore, the gels formed with LMP are thermo-irreversible in the presence of calcium and stay gelled upon heating at high temperatures which would normally melt the gel (Chan, et al., 2016). In addition, amidated pectin can be obtained when HMP is hydrolysed using ammonia converting some of the ester groups into amide groups (May, 1990). Amidation influences the properties of the gel formed by pectin. Amidated pectin is more pH sensitive and, thus, acid induced gelation is more favourable (Capel, Nicolai, Durand, Boulenguer, & Langendorff, 2006; Chan, et al., 2016). This is due to the fact that the presence of hydrogen bonds between the amide groups helps promoting the formation of a gel (Chan, et al., 2016). Furthermore, amidated pectin enables the production of thermoreversible gels which cannot be achieved by conventional pectin (Chen, et al., 2015). The selection of pectin to be used in different food applications depends on the desired physical characteristics of the products, the presence of proteins, molecular weight, pH, and processing temperature (Walter, 1991).

Pectin works very well as a gelling, emulsifying, stabilizing and thickening agent (Chan, Choo, Young, & Loh, 2016). Pectin contains a very limited amount of protein in its structure. Thus, limited adsorption of pectin at the oil and water interface is possible (Alba & Kontogiorgos, 2016). As thickening agent, the long term stability of the emulsions in which pectin was added is attributed to the thick adsorbed layers which provide steric stabilization and prevent the emulsion from coalescence. Studies showed that the steric stabilization of pectin is attributed to the hairy rhamnogalacturonan I (RG-I) part of the pectin while the electrostatic stabilization is attributed to the homogalacturonan (HG) part of the pectin backbone due to ionization of the carboxylic groups since at pH above 3.5 they are ionized (Alba & Kontogiorgos, 2016; Chan et al., 2016).

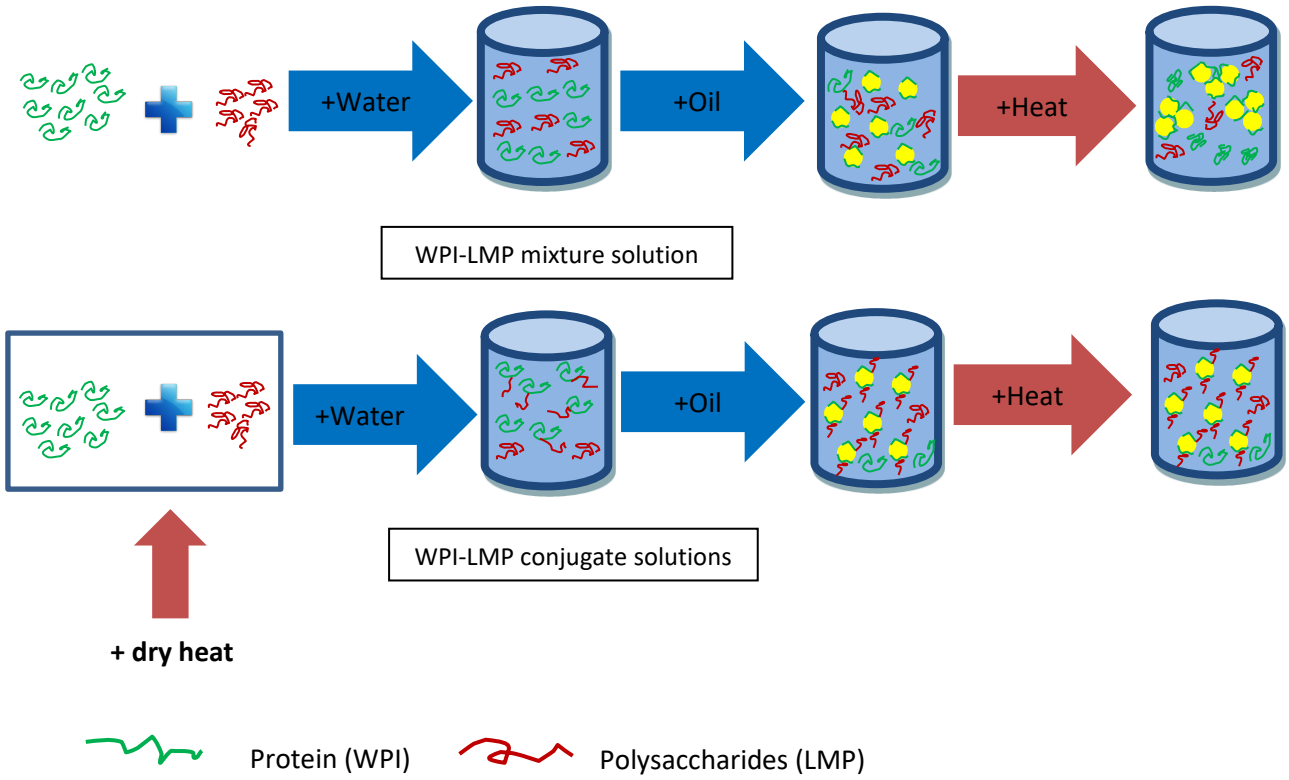
1.10 Objectives

The general objective of this PhD research was to improve the heat stability of whey proteins through dry heat treatment with pectin. Improving heat stability of whey proteins is of utmost importance to broaden their application in food industry. This is due to the fact that heat treatment is inevitable in food processing. In dairy industry, heat induced denaturation of whey proteins is one of the main cause of fouling. Fouling can reduce the heat transfer efficiency of the plate heat exchanger and induce microbial growth, which are highly undesirable. This is caused by the interaction of denatured whey proteins and caseins micelles. It was suggested that this event can be avoided by improving the heat stability of whey proteins. Hence, improving heat stability of whey proteins will also benefit the milk industry.

Heat induced whey proteins denaturation and aggregation are detrimental to the quality of the food products. These phenomena lead to an increase in the viscosity of the products and thus change in consistency. In addition to the heat stability of the conjugates, the functionality of the conjugates, especially their emulsifying activity was also evaluated. It is important that the whey proteins are able to maintain their functionality after dry heat treatment with pectin. Furthermore, this study also aimed to study the factors that influence the performance of the conjugates in stabilizing emulsions against gravitational separation and heat induced aggregation.

CHAPTER 2

IMPROVED HEAT STABILITY OF PROTEIN SOLUTIONS AND O/W EMULSIONS UPON DRY HEAT TREATMENT OF WHEY PROTEIN ISOLATE IN THE PRESENCE OF LOW-METHOXYL PECTIN



Redrafted from: Setiowati, A. D., Vermeir, L., Martins, J., De Meulenaer, B., & Van der Meeren, P. (2016). Improved heat stability of protein solutions and O/W emulsions upon dry heat treatment of whey protein isolate in the presence of low-methoxyl pectin. Colloids and Surfaces A: Physicochemical and Engineering Aspects, Colloids and Surfaces A: Physicochemical and Engineering Aspects, 510, 93-103.

Abstract

Whereas whey protein isolate may be used as effective emulsifier, it suffers from limited stability upon heating. In this research, the effect of combining whey protein isolate (WPI) with low methoxyl pectin (LMP) on the heat stability of WPI was investigated. This was accomplished either by simple mixing or by conjugate formation by dry heat treatment. WPI-LMP mixtures and conjugates were prepared at a WPI to LMP ratio of 1:0, 4:1, 2:1, and 1:1. Conjugates were prepared by means of dry heat treatment at a temperature of 60^{o}C and 74% relative humidity by incubation for up to 16 days.

The pH effect and brown colour development of the WPI-LMP conjugates upon incubation was monitored. SDS-PAGE, free amino group determination, as well as diffusion coefficient analysis, all confirmed the formation of new compounds with high molecular weight. The heat stability of the conjugates was then tested and compared to the native WPI. Upon 2 minutes of heating at 80 ^{o}C and pH 6.5, the solubility of WPI was reduced by approximately 50% due to thermal denaturation and subsequent aggregation. However, dry heat treatment of the WPI-LMP mixtures highly improved the heat stability of WPI: as the incubation time was increased, the residual protein solubility upon heating of the WPI became higher. Upon 16 days of incubation, the protein solubility of the heated WPI-LMP conjugates was comparable to that of the conjugates before heating. Considering the emulsifying properties, it was found that WPI-LMP conjugates produced smaller oil droplet compared to either native WPI, dry heated WPI, or mixtures of WPI-LMP which were not subjected to conjugation by dry heat treatment. Heating the emulsions at 80 ^{o}C for 10 and 20 minutes revealed that WPI-LMP conjugates stabilized emulsions exhibited excellent stability towards heat: whereas pronounced aggregation and gelation occurred in emulsions stabilized by WPI or mixtures with LMP, the conjugate stabilized emulsions retained their original viscosity and particle size.

Keywords: Whey Protein Isolate, Low Methoxyl Pectin, Dry heat treatment, Conjugate, Heat Stability, Emulsion.

2.1 Introduction

Whey Protein Isolate (WPI) is known to have excellent emulsifying properties. However, this dairy protein source exhibits a low stability towards heat and is very susceptible to heat denaturation (Jovanović, et al., 2005) which can lead to partial protein denaturation and aggregation, thus affecting its emulsifying properties (Raikos, 2010). As heat processing is commonly encountered in the dairy industry for improving the safety and shelf life of foods (Wijayanti, Bansal, & Deeth, 2014), the limited heat stability of WPI becomes a limiting factor for the application of WPI on an industrial scale (Dissanayake, Ramchandran, Donkor, & Vasiljevic, 2013b). Several researches on improving the heat stability of WPI have been performed using different approaches. Chemical methods (Demetriades & McClements, 2000; Hoffmann & van Mil, 1997), enzymatic modification (Chobert, Bertrand-Harb, & Nicolas, 1988; Gauche, Vieira, Ogliari, & Bordignon-Luiz, 2008), electrostatic interactions (Harnsilawat, et al., 2006a; Jones, et al., 2009), and conjugation by heat (Jiménez-Castaño, Villamiel, et al., 2005) are among the methods that have been used. Some of the methods showed a successful improvement of the heat stability and emulsifying properties of WPI (Dickinson & Semenova, 1992; Diftis, et al., 2005; Kato, Sasaki, Furuta, & Kobayashi, 1990; Vardhanabhuti, et al., 2008). Nevertheless, among the methods mentioned, chemical methods involve chemicals which sometimes are not compatible for food application. On the other hand, electrostatic interaction has a narrow range of optimum pH and is sensitive to the presence of electrolytes which limits its application (Dickinson, 1993a). Conjugation by heat is a promising method as it does not involve any chemical addition. This method relies on the addition of another biopolymer to be conjugated to the WPI.

It is known that polysaccharides have a good stabilizing activity (Dickinson, 2009). It was stated that the presence of a biopolymer can influence or modify the functional properties of other biopolymers when they are mixed (Harnsilawat, et al., 2006a). Thus, combining the properties of WPI and polysaccharides can be a good way to modify the properties of WPI resulting in a molecule with improved functionality (Dickinson & Galazka, 1991). It has been reported that the presence of polysaccharides enables WPI to minimize structural losses during heating due to the steric force obtained from the polysaccharides (Dickinson, 1993a; Dickinson, et al., 1992; Xia, et al., 2015).

Heat induced conjugation of WPI and polysaccharides results in hybrid molecules which are linked together by covalent bonds. This type of interaction is found to be more heat stable than electrostatic interaction (Dickinson & Galazka, 1991; Stainsby, 1980). Conjugation of WPI and polysaccharides by heat can be performed either in wet state (Diftis, et al., 2005; Zhu, et al., 2010) or in dry state (Diftis & Kiosseoglou, 2006a; Kato, et al., 1990; Martinez-Alvarenga, et al., 2014b). The latter is more preferable since it is easier to handle dry matter and also microbial contamination leading to an unstable product which can occur in the wet state can be avoided (Oliver, et al., 2006). As this method does not require any chemical addition, it can be a promising replacement for synthetic surfactants in food applications (Garti, 1999b), whereby it is suitable for the production of "clean-label" emulsions. Another advantage is that the dry heat treatment does not significantly alter the native-like behaviour of the protein while wet heat treatment causes important structural changes resulting in a specific denatured β-lactoglobulin monomer, which is covalently associated via the free thiol group (Morgan, et al., 1999).

Conjugation of WPI and polysaccharides through dry heating can be performed by exposing the dry mixture of WPI and polysaccharides to heat in an atmosphere of controlled relative humidity for a certain period of time. Depending on the type of the protein and polysaccharides used, the incubation can take from hours to days. It is suggested that upon dry heating, Maillard type reactions are responsible for the formation of the conjugates. Conjugation of protein and pectin is basically based on the Amadori arrangement which is part of the Maillard type reaction (Oliver, et al., 2006). Hereby, the terminal and side chain amine groups of the protein are linked to the reducing end of the polysaccharides, resulting in a conjugate of protein and polysaccharides (Dickinson, 2008). However, it has to be kept in mind that the advanced stage of the Maillard reaction is undesirable since it can lead to a loss of solubility, and hence a reduction of the functionality of the WPI (Jiménez-Castaño, Villamiel, et al., 2005; Kato, 2002). Therefore, the challenge is to obtain WPI-polysaccharide conjugates without any loss of functionality of the WPI during heat treatment (Dickinson, 1995).

The purpose of this research was to improve the heat stability of WPI by grafting low methoxyl pectin (LMP) to the WPI via dry heat treatment. Pectin was chosen due to its abundant availability which makes it a cheap source of polysaccharides. Furthermore, polysaccharides

are more desirable than oligosaccharides and monosaccharides because they are less prone to advanced stages of Maillard reaction that lead to loss of the protein solubility (Kato, 2002). The WPI was mixed with the LMP at various ratios and dry heating was performed at 60^{o}C and 74% RH. Various experiments were performed to confirm the formation of WPI-LMP conjugates and to study the heat stability of the obtained conjugates.

2.2 Materials and Methods

2.2.1 Materials

The WPI was purchased from Davisco Foods International Inc. (Le Sueur, MN, USA). Protein analysis revealed that the WPI contained approximately 92.6% protein, whereby 85% of the protein is β-lactoglobulin (Van der Meeren, El-Bakry, Neirynck, & Noppe, 2005). The low methoxyl pectin (LMP) (UnipectinOB700) was obtained from Cargill (Ghent, Belgium) and contained 89.6% of dry matter. The LMP was used without further purification. Oil in water emulsions were prepared using sunflower oil purchased from a local supermarket as the oil phase.

2.2.2 WPI-LMP conjugate preparation

Conjugates were prepared from a 5% (w/v) protein solution and 1% (w/v) LM Pectin solution. A correction for the protein content and the dry matter was taken into account during the calculation of the WPI and LMP needed. The WPI and LMP were dissolved in distilled water and the pH of the solutions was adjusted to 7.0 with 1 N HCl to avoid formation of ionic complexes that might form at lower pH during mixing. Both solutions were kept overnight in a refrigerator before mixing. The solutions were then mixed at four different WPI to LMP ratios i.e. 1:0, 4:1, 2:1, and 1:1 (on weight basis) and frozen prior to freeze drying.

The frozen samples were lyophilized (Alpha 1-2 LD plus, Christ) to remove all the water and obtain dry products. The freeze dried products which were placed in a container with a diameter of 52 mm were then incubated at a temperature of 60^{o}C for 16 days in a desiccator containing saturated NaCl solution to keep the relative humidity at 74% (Greenspan, 1977). During incubation, sampling was done at day 4, 8, and 16.

2.2.3 pH and absorbance measurement

A 2.0 mg/mL WPI-LMP conjugate solution was prepared by dissolving the conjugates in distilled water. The pH and brown colour development of the solutions were then measured without further dilution. The pH was measured using a Hanna H 4222 pH meter**,** while the brown colour development was measured at 420 nm (Sun, Yu, Zeng, Yang, & Jia, 2011)using a UV-1600 PC, spectrophotometer (VWR).

2.2.4 SDS-PAGE Analysis

The SDS-PAGE analysis was performed under reducing conditions in the presence of mercaptoethanol. The running gel and stacking gel contained 15% and 4% of polyacrylamide. Conjugates were diluted in a 20 mM sodium phosphate buffer (pH 6.5) to a concentration of 1.0 mg protein/mL. The solutions were then diluted in Laemlli buffer which contained mercaptoethanol to obtain a concentration of 0.5 mg/mL. These diluted solutions were then heated at 90^{o}C, followed by centrifugation at 10000 rpm for 5 minutes. Subsequently, 20 µL of the solution was injected in to the gel. The electrophoresis was performed at 160 V for at least 1 hour and 15 minutes. The gel was subsequently stained using Coomassie blue to visualize the protein.

2.2.5 High resolution pfg-NMR diffusometry

High-resolution pulsed field gradient (pfg) NMR diffusion analysis was performed with a Bruker Avance III spectrometer operating at a ^{1}H frequency of 500.13 MHz and equipped with a 5 mm DIFF30 gradient probe with a maximum gradient strength of 18 T/m. Pfg-NMR experiments were performed at room temperature using a monopolar (single) stimulated echo pulse sequence. The samples (650 µL) were filled in 5 mm diameter glass NMR tubes (Armar Chemicals, Switzerland) and were measured upon varying the gradient strength up to 12 T/m, while keeping the gradient duration (δ) constant at 1 ms and the diffusion delay (Δ) fixed at 100 ms. The (non-conjugate) powders of WPI (dry heated for 16 days) and LMP were dissolved in 5 mM sodium acetate (in D_2O) to obtain a concentration of 10 mg/mL, whereas WPI-LMP conjugates were dissolved in 5 mM sodium acetate to obtain a concentration of 20 mg/mL.

Regarding the non-conjugate WPI (WPI dry heated for 16 days) and LMP samples, the obtained experimental echo attenuation ratio (I/I_o) with up to 95% decay as a function of gradient strength was fitted by Eq. 2.1a and Matlab 7.5.0.342 (R2007b) software (The Mathworks). Hereby, a mass-weighted lognormal distribution of diffusion coefficients was assumed.

$$\left(\frac{I}{I_0}\right)_{expt} = \int_0^{\infty} P_v(D_i) \cdot \frac{I}{I_0}(D_i) \cdot \, dD_i \qquad \text{(Equation 2.1a)}$$

$$\frac{I}{I_0}(D_i) = \exp\left(-D_i \cdot \gamma^2 \cdot G^2 \cdot \delta^2 \cdot \left(\Delta - \frac{\delta}{3}\right)\right) \qquad \text{(Equation 2.1b)}$$

$$P_v(D_i) = \frac{1}{\sqrt{2\pi} \cdot D_i \cdot \ln \sigma_g} \cdot \exp\left(-\frac{\left(ln(D_i) - ln\left(D_g\right)\right)^2}{2 \cdot \left(\ln \sigma_g\right)^2}\right) \qquad \text{(Equation 2.1c)}$$

$$D_a = D_g \cdot \exp\left(\frac{\left(\ln \sigma_g\right)^2}{2}\right) \qquad \text{(Equation 2.1d)}$$

$$\sigma = \sqrt{{D_a}^2 \cdot \left(exp\left(\left(\ln \sigma_g\right)^2\right) - 1\right)} \qquad \text{(Equation 2.1e)}$$

Protein and pectin samples may be characterized by a molecular mass range and hence, a polydisperse population of diffusion coefficients with a certain probability P_v. In our calculation, P_v was assumed to follow by a lognormal mass-weighted diffusion coefficient distribution (Eq. 2.1c). The geometric mean diffusion coefficient (D_g) and geometric standard deviation (σ_g) were converted to the arithmetic mean diffusion coefficient (D_a) and arithmetic standard deviation (σ) of the lognormal mass-weighted diffusion coefficient distribution using Eq. 2.1d and Eq. 2.1e.

The degree of molecular interaction can be evaluated upon decomposing the WPI diffusion signal obtained from the WPI-LMP conjugate sample $\left[\frac{I}{I_0}\right]_{WPI,conjug.}$ into a freeWPI fraction (i.e. the experimentally obtained non-conjugate WPI signal $\left[\frac{I}{I_0}\right]_{WPI,free}$) and a bound WPI fraction. The bound fraction can be determined upon estimating the diffusion signal of the reacted WPI $\left[\frac{I}{I_0}\right]_{WPI,bound}$ using Eq. 2.2 and Matlab 7.5.0.342 (R2007b software, The Mathworks). As the

molar mass of pectin is much larger as compared to WPI, the molar mass of the conjugate is mostly determined by the pectin. Hence, the bound fraction can be determined assuming the experimentally obtained LMP diffusion signal to be a good approximation of the LMP-bound WPI diffusion signal as written down in Eq. 2.3 using the Solver add-in (Microsoft Excel 2010). A higher value of the coefficient of determination was obtained upon fitting Eq. 2.1 to the conjugate LMP signal $\left[\frac{I}{I_0}\right]_{LMP,conjug.}$ (R^2=0.999 in Table 2.1) as compared to the non-conjugate LMP (R^2=0.996) as recorded at 3.2-4.2 ppm. Therefore, the former signal was used in Eq. 2.3, for which D_a and σ amounted to $5.4 \cdot 10^{-11}$ m^2/s and $15 \cdot 10^{-11}$ m^2/s.

$$\left[\frac{I}{I_0}\right]_{WPI,conjug.} = \varphi_{free} \cdot \left[\frac{I}{I_0}\right]_{WPI,free} + \varphi_{bound} \cdot \left[\frac{I}{I_0}\right]_{WPI,bound} \quad \text{(Equation 2.2)}$$

$$\left[\frac{I}{I_0}\right]_{WPI,conjug.} = \varphi_{(1-\varphi_{bound})} \cdot \left[\frac{I}{I_0}\right]_{WPI,free} + \varphi_{bound} \cdot \left[\frac{I}{I_0}\right]_{LMP,conjug.} \quad \text{(Equation 2.3)}$$

2.2.6 TNBS Analysis for Free Amino Group Determination

TNBS analysis measures the amount of free amino groups in the sample. This analysis was carried out to find the degree of graft reaction. By knowing the available amino groups in the sample, the approximate amount of amino groups that are cross-linked with the polysaccharide during dry heat incubation can be estimated and hence the degree of the graft reaction can be obtained.

The analysis was performed based on the method developed by Adler-Nissen (1979). All samples, blank, and standards were dissolved in 1% SDS solution prior to analysis. 0.25 mL of each sample was brought into a test tube. Subsequently, 2.0 mL of sodium phosphate buffer (0.2125 M, pH 8.2±0.02) and 2.0 mL of a 0.1% TNBS solution were added. The test tube was shaken with a vortex and placed in a water bath at 50°C for 60 minutes. The water bath should be covered to avoid light penetrating into the test tubes since light can accelerate the reaction in the blank solution. Afterwards, 4.0 mL of 0.1 N HCl was added to terminate the reaction. The test tubes were then allowed to cool down to room temperature. The absorbance of the samples was read at 340 nm in a spectrophotometer (UV-1600 PC, VWR). A standard curve

was obtained by considering a dilution series of leucine in sodium phosphate buffer with concentrations up to 2 mM. The amount of available amino groups (mM) was then deduced from the standard curve. The degree of graft reaction was calculated as:

$$degree\ of\ graft\ reaction\ (DG) = \frac{(-NH_2\ in\ mixture(day\ 0) - -NH_2\ in\ conjugates\ (day\ x))(mM)}{-NH_2\ in\ mixture(day\ 0)\ (mM)}\ x\ 100 \qquad \text{(Equation 2.4)}$$

2.2.7 Heat treatment

2 mL of solution containing conjugates (1% w/v) were prepared for heat treatment. The conjugates were dissolved in Imidazole buffer containing 20mM imidazole, 30 mM NaCl, and 1,5mM NaN_3; the pH of the buffer was adjusted to 6.55 using 1 N HCl. Subsequently, the solutions were heated at 80°C for 2 minutes using a water bath. Samples were placed into cold water after the heat treatment to stop denaturation. Heat treated samples were then put into 2 mL centrifugation tubes and centrifuged (Sigma, Scientific 1-15 P) at 12000 g for 20 minutes to separate the insoluble aggregates. The supernatant was kept for further analysis.

2.2.8 Colorimetric protein determination

A modification and simplification of the Lowry analysis method by Schacterle and Pollack (Schacterle & Pollack, 1973) was used. Prior to analysis, samples were diluted in imidazole buffer of pH 6.55 to fit in the range of the standard curve. 1 mL of sample was brought into a test tube in which 1 mL of alkaline Cu-reagent was added. This step was followed by mixing the solution using a vortex and leaving the solution undisturbed for 10 minutes. Subsequently, 4.0 mL of Folin Ciocalteu reagent was added and the tube was turned upside down two times. The solution was heated at 55°C for 5 minutes using a water bath. The heated solution was put in ice-water immediately after heating. Subsequently, the absorbance of the solutions was read at 650 nm against a blank solution in a spectrophotometer (VWR, UV-1600 PC). A series of standard solutions was made by dissolving WPI in imidazole buffer to a concentration of 0, 40, 80, 120, 160, 200, 240 mg protein/L. A correction for the protein content in WPI should be performed when calculating the amount of WPI needed in the standard solution.

2.2.9 Emulsion preparation

Emulsions were initially prepared by dissolving 0.5% of WPI, dry heated WPI, mixture of WPI-LMP, and WPI-LMP conjugates in the aqueous phase. The aqueous phase was kept overnight in the fridge prior to the emulsion preparation to fully hydrate the hydrocolloid. Emulsions containing 10% (w/w) of oil were prepared by adding 10 g of sunflower oil to 90 g of WPI solution.

The mixture was then premixed using an IKA Ultra-turrax TV45 (Janke & Kunkel, Staufen, Germany) at the highest speed (24000 rpm) for 1 minute. This was followed by homogenization using a Microfluidizer110S for 2 minutes at 4 bar of compressed air pressure corresponding to 560 bar of liquid pressure. The coil of the Microfluidizer was immersed in a water bath set at 55^{o}C. The heat coagulation test was performed by heating the emulsions in an oil bath at 80^{o}C for 10 and 20 minutes.

2.2.10 Particle Size analysis

Particle size analysis was performed using a Mastersizer 3000 (Malvern Instrument Ltd, Malvern, UK) equipped with a red and blue light source. The refractive index used was 1.47, while the absorbance index was set at 0.01. The sample was added drop wise to the wet sample distribution unit (Malvern Hydro MV) until an obscuration level between 10 to 20% was obtained. The speed of the stirrer of the dispersion unit was set at 1500 rpm during the measurement.

2.2.11 Statistical Analysis

Two-way ANOVA was performed on the results of protein solubility analysis using SPSS 22 (IBM) at a significance level of 95%.

2.3 Results and Discussion

2.3.1 Confirmation of Conjugate formation upon incubation of WPI and LMP

Conjugation between protein and pectin is possible by exposing the two components to heat in a controlled environment. When proteins and polysaccharides are mixed and exposed to heat, the Maillard reaction will take place spontaneously. Upon incubation of WPI and LMP,

as the Maillard reaction proceeds there will be changes in the environment of the mixture. This can be used as an indicator to monitor the Maillard reaction and to confirm the formation of WPI-LMP conjugates.

2.3.1.1 pH change and brown colour development

Figure 2.1 shows the pH of the WPI-LMP conjugates as a function of the incubation time. The initial pH of the WPI and LMP dissolved in distilled water was approximately 6.6 and 5.0, respectively. However, the pH of the WPI and LMP solutions was adjusted to 7 before mixing to avoid electrostatic interactions. After freeze drying, the pH of the reconstituted dry mixture of WPI-LMP was found to be between 6.4-6.5. During dry heat incubation, the pH of all the conjugates, regardless of the mixing ratio, decreased. During Maillard reaction, primary amino groups will react and intermediate products are generated, which may contain acidic compounds (Chen, Jin, & Chen, 2005; DeMan, 1976; Liu, Yang, Jin, Hsu, & Chen, 2008), which can explain the pH depression of the conjugates upon incubation. Overall, the pH decreased mainly in the first 4 days. It was found that there was no significant effect of dry heat treatment in the absence of LMP. In general, the more LMP was present in the mixture the lower the pH of the conjugates became. It was reported before by Liu et al (2008) that due to Maillard reaction, the pH will decrease linearly with time. However, on systems with higher sugar content and incubated at 90^{o}C the trend was different: the pH decreased fast during the first few hours and upon further incubation it decreased at a slower rate (Liu, et al., 2008). This trend is similar to the trend of the conjugates with a ratio of 2:1 and 1:1 (Fig. 2.1).

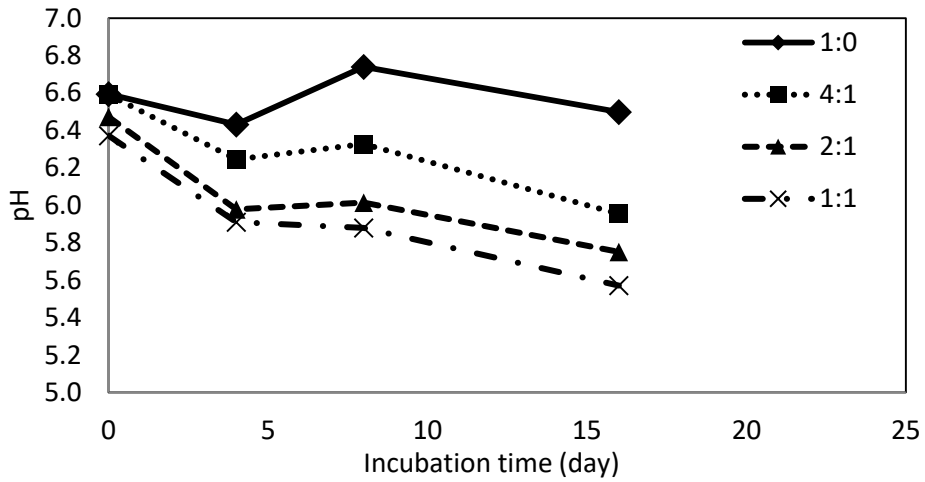


Figure 2.1 pH of a 2 mg/ml solution of the WPI-LMP conjugates (WPI to LMP ratio of 1:0, 4:1, 2:1, and 1:1) as a function of incubation time.

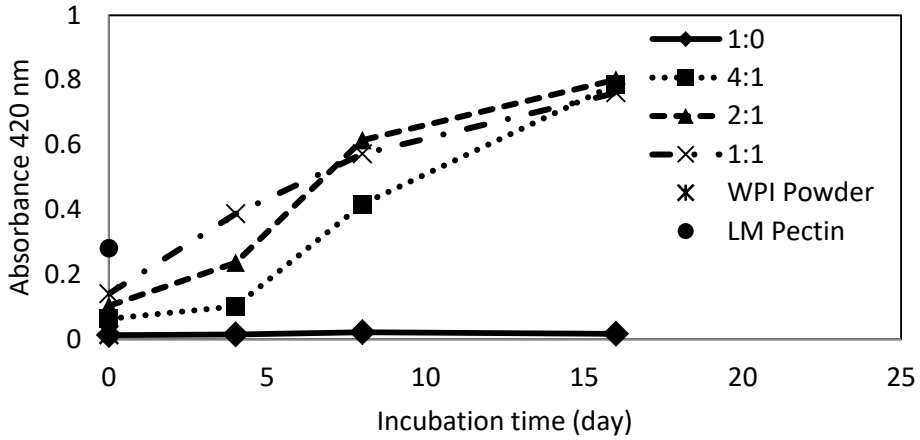


Figure 2.2 Brown colour development of WPI-LMP conjugates (WPI to LMP ratio of 1:0, 4:1, 2:1, and 1:1) as a function of incubation time, measured in a solution containing 2mg/ml of conjugates.

It is known that the Maillard reaction results in brown pigments. Therefore, the progress of the Maillard reaction can be followed by the formation of these pigments in the dry mixture of WPI-LMP. Considering the absorbance at 420 nm as a function of incubation period of the conjugates (Figure 2.2) it can be clearly seen that brown pigment formation became more intensive as the incubation time was extended. In agreement with the pH results, the dry heated WPI (Ratio 1:0) had a stable colour and did not undergo development of brown colour.

In a similar way, the formation of intermediate products during the Maillard reaction can be evaluated from the absorbance at 294 nm. Comparing the absorbance readings at 294 (data not shown) and 420 nm, it was seen that the formation of the intermediate products had the same pattern as the formation of the brown colour in the conjugates. This result is in agreement with the finding of Lertittikul et al. (2007). It can be clearly seen in Figure 2.2 that the more LMP was present in the conjugates, the higher was the initial rate of brown colour formation during incubation. On the other hand, after 16 days all conjugates had a comparable absorbance value. Based on the pH change and brown colour development, it can be stated that the Maillard reaction took place upon incubation of the WPI-LMP mixture.

2.3.1.2 TNBS Analysis

According to Oliver et al. (Oliver, et al., 2006), Maillard reaction is a spontaneous and naturally occurring reaction between available amino groups from a protein with reducing sugar

entities from polysaccharides, which involves the Amadori rearrangement (Dunlap & Côté, 2005; Jiménez-Castaño, Villamiel, et al., 2005). Thus, during the Maillard reaction amino groups of the protein will be consumed and as a consequence the availability of the amino groups decreases. This phenomenon can be used as an indicator to observe the Maillard reaction taking place during incubation. By knowing the amount of the free amino groups in the conjugates at different incubation times, the degree of glycosylation or graft reaction during incubation can be estimated.

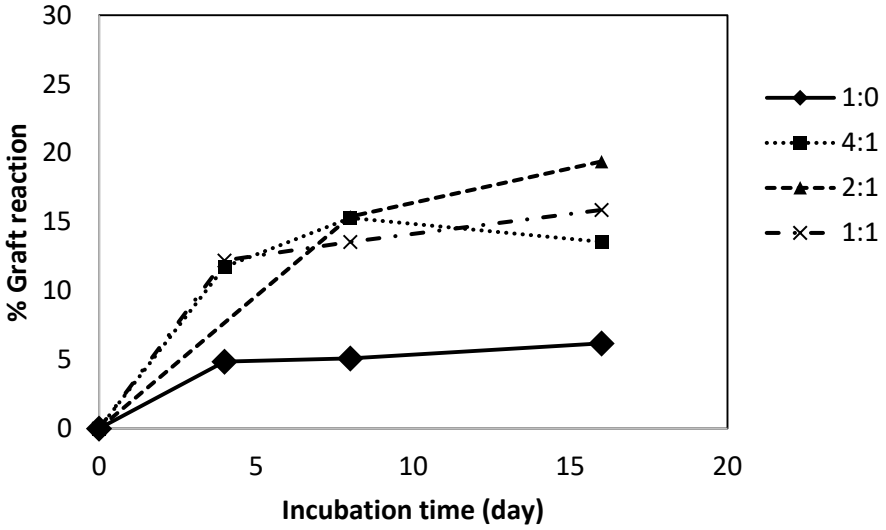


Figure 2.3 Degree of graft reaction of the dry heated WPI (ratio 1:0) and conjugates of WPI-LMP (ratio 4:1, 2:1, and 1:1) during incubation at 60°C and RH of about 74%.

Figure 2.3 shows that the free amino group content of dry heated WPI was reduced which could be due to protein polymerization upon dry heat treatment. For WPI-pectin conjugates, the free amino group content decreased by about 15% on the 8th day of the incubation.

From Figure 2.3, it can be depicted that the rate of the graft reaction was most pronounced during the initial period of the incubation, and gradually decreased later on. The latter could be due to the steric hindrance of the LMP which was already attached to WPI. Upon dry heating of BSA with dextran, it was found that a steady state reaction was obtained due to the hindrance from the dextran attached in the BSA (Xia, et al., 2015).

It should be kept in mind that a reduction of the free amino group content in the samples does not necessarily mean an enhancement of the graft reaction. It was reported before that

polymerization of protein occurred during incubation of β-Lactoglobulin and dextran at a temperature of 55°C, a_w of 0.65 and 6:1 weight ratio (dextran to β-Lactoglobulin) (Dunlap, et al., 2005; Jiménez-Castaño, Villamiel, et al., 2005). This polymerization reaction may reduce the ability of the free amino groups to react with the TNBS leading to lower absorbance readings at 420 nm. Hence, the reduction of free amino group content observed was probably not solely due to glycosylation of whey protein, but could be the result of protein polymerization as well.

2.3.1.3 SDS-Page analysis

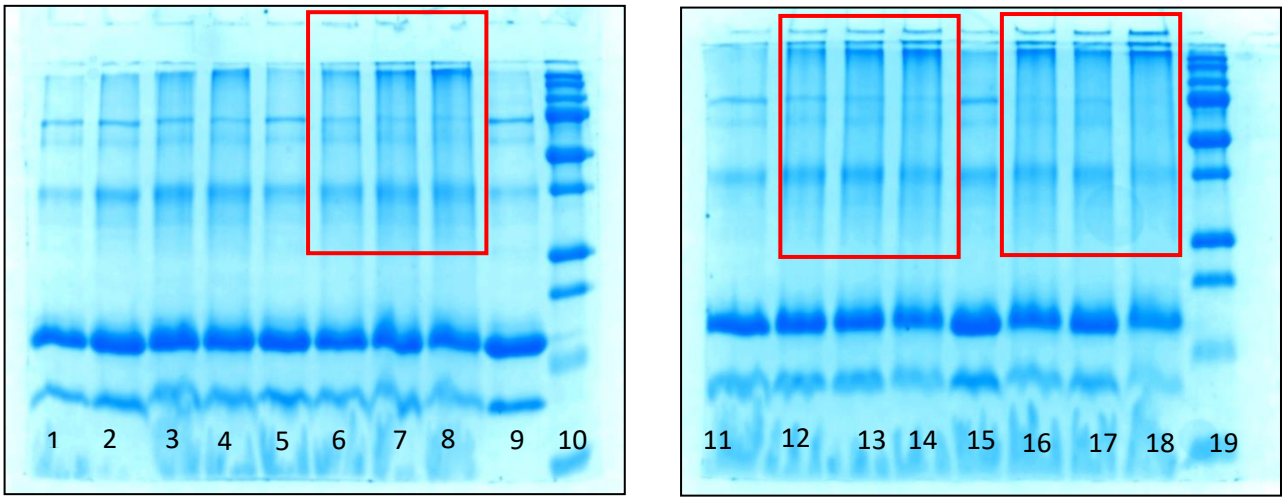


Figure 2.4 SDS-PAGE results of WPI and WPI-LM Pectin Conjugates incubated at 60°C in an environment containing saturated NaCl solution, with different ratios of WPI to LM Pectin (Lanes: 1, 2,3,4, Conjugate ratio 1:0; 5,6,7,8,conjugate ratio 4:1; 9, WPI powder; 10, molecular weight marker; 11, 12, 13, 14, conjugate ratio 2:1; 15,16,17,18, conjugate ratio 1:1) incubated for different periods (Lanes: 1,5,11,15, Day 0; 2,6,12,16, Day 4 days; 3,7,13,17, Day 8; 4,8,14,18, Day 16).

Figure 2.4 shows the scanned gel from the SDS-PAGE analysis. For comparison, WPI without any treatment was included as well (Lane 9). The gel profile shows that WPI had two distinct bands, which could be correlated to β-lactoglobulin and α-lactalbumin. In the upper part or higher molecular weight part of the gel it could be seen that there were bands which represented the dimer of β-lactoglobulin and BSA. The molecular weight of β-lactoglobulin, α-

lactalbumin, dimer of β-lactoglobulin and BSA is approximately 18.4 kDa, 14 kDa, 36.8 kDa, and 66.5 kDa, respectively.

In Fig. 2.4, it can be seen that the mixtures which were only lyophilized but not heat treated (indicated as 0 days) of WPI-LMP for all ratios had the same band pattern as the WPI powder. As the mixtures were incubated, the band for α-lactalbumin seemed to be fading as the incubation period was extended. Furthermore, there were broad bands of high molecular weight compounds in the lanes of the conjugates incubated for 4, 8, and 16 days. The intensity of the band of these high molecular weight compounds increased with the duration of the incubation time. Furthermore, in the injection point of these samples it could also be observed that there was a band representing compounds which were trapped due to their big molecular weight (Lane 12, 13, 14, 15, 16, and 17). In the same lanes there were also compounds trapped in between the stacking and running gel due to their big molecular weight. This leads to the conclusion that the conjugation of WPI and LM Pectin clearly produced compounds with high molecular weight.

Nevertheless, in the dry heated WPI (ratio 1:0) incubated for 4, 8, and 16 days there was also an intensification of the bands of high molecular weight molecules which showed that there was polymerization of the WPI during incubation. It was reported before that dry heating of β-lactoglobulin resulted in compounds with MW higher than the MW of its dimer (Jiménez-Castaño, Villamiel, et al., 2005). This polymerization can be caused by covalent bond formation by either disulphide bonds or other types of covalent bonds (Dickinson & Galazka, 1991; Enomoto, et al., 2009; Gulzar, et al., 2011; Jiménez-Castaño, López-Fandiño, et al., 2005). Heating will unfold the protein, whereby sulfhydryl containing amino acid groups will be exposed. Particularly, in β-lactoglobulin, sulfhydril-disulphide interchange is initiated when the protein is heated at temperatures between 60-65^{o}C (Prabakaran & Damodaran, 1997). Since the WPI used is mainly composed of β-lactoglobulin, it is reasonable to compare the behaviour of WPI during heating to that of β-lactoglobulin.

Whereas disulphide bonds in the protein aggregates should have been eliminated due to the presence of mercaptoethanol, still protein aggregates with high molecular weight were observed in the lane of the dry heated WPI. This suggests that besides disulphide bonds, other covalent bonds were responsible for the polymerization in the dry heated WPI. Upon dry

heating of WPI at 100 ^{o}C and different pH conditions, Gulzar, et al. (2011) found that at low pH protein aggregation was mainly due to intermolecular disulphide bonds, while as the pH increased to 6.5, protein aggregation was due to disulphide bonds and covalent bonds other than disulphide bonds. The latter observation is in line with our experiment as the pH of the dry heated WPI was approximately 6.5 (Fig. 2.1).

Due to this phenomenon, it can be questioned whether the high molecular weight compounds observed in the WPI-LMP conjugates were partly because of protein aggregation. In the case of WPI-LMP conjugates (ratio 4:1, 2:1, and 1:1), however, this phenomenon could be minimized or prevented due the presence of LMP. Several authors observed that conjugation of proteins and polysaccharides through Maillard reaction can minimize the structural loss of the protein by protein aggregation during dry heat treatment (Spotti, et al., 2014; Sun, Hayakawa, & Izumori, 2004; Xia, et al., 2015). In fact, aggregation and Maillard reaction can happen simultaneously and it was reported previously that BSA aggregation upon dry heating can be reduced in the presence of dextran since it can prevent extra aggregation of BSA (Xia, et al., 2015). Furthermore, the author suggested that in the presence of dextran, the amino acids containing the sulfhydryl group responsible for protein aggregation via disulphide bond formation are involved in the Maillard reaction, thus preventing aggregation between proteins. Hence, it is expected that protein aggregation was probably very limited in the presence of LMP.

Whereas it is difficult to use SDS-PAGE results for quantification of the degree of conjugation, still the results can be used as a confirmation of the formation of high molecular weight conjugates during dry heat incubation of WPI and LMP. This result is in agreement with the results of TNBS analysis, the results of pH change and colour development analysis which indicated that Maillard reaction already occurred during the first 4 days of the incubation. In addition, SDS-PAGE analysis showed that there was protein aggregation during dry heat treatment of WPI alone which is thought to involve the consumption of free amino groups. This finding was supported by the fact that TNBS analysis showed a reduction of free amino group content of the WPI upon dry heat treatment of WPI.

2.3.1.4 Diffusion analysis via NMR analysis

Further analysis to confirm the formation of conjugates was performed using high resolution pfg-NMR diffusometry. This measurement is based on the diffusivity measurement of the targeted compound. The analysis was performed on WPI-LMP conjugates with a ratio of 1:1 which were incubated for 16 days. In order to know the diffusion behaviour of WPI and LMP alone, diffusion NMR was performed on solutions of LMP separately as well as lyophilized WPI which was incubated for 16 days.

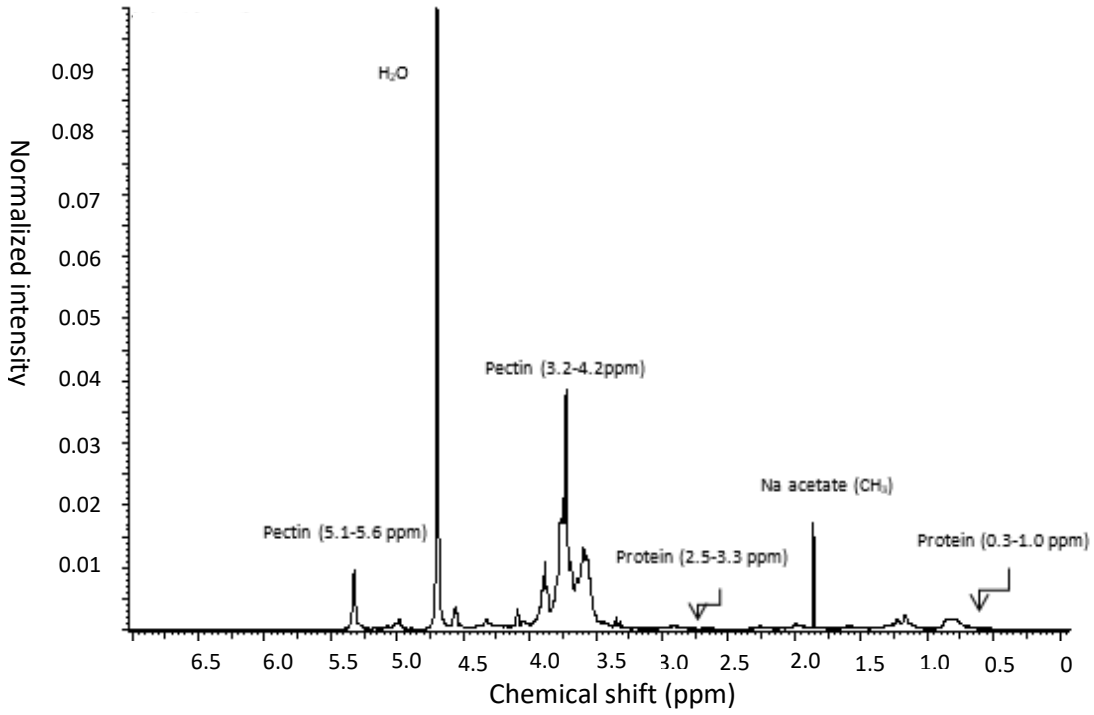


Figure 2.5 1D ^{1}H spectrum of WPI-LMP conjugates (ratio 1:1) upon dry heat incubation for 16 days.

Figure 2.5 shows the 1D ^{1}H spectrum of the WPI-LMP conjugates of ratio 1:1 which was incubated for 16 days. By comparing this spectrum to the spectra of WPI and of LMP, it became obvious that the signals observed at 0.3-1.0 ppm and 2.5-3.3 ppm belonged to the WPI, while the spectrum at 3.2-4.2 ppm and 5.2-5.5 ppm belonged to the LMP. The other sharp resonances observed represented the H_2O and the sodium acetate added upon sample preparation. By fitting equation 2.1a, the arithmetic mean diffusion coefficient and the arithmetic standard deviation of each signal were obtained (Table 2.1). There was one major signal (0.3-1.0 ppm) and one minor peak (2.5-3.3 ppm) detected for WPI and both had

approximately the same diffusion coefficient ($6.9 \cdot 10^{-11} m^2/s$) and a narrow distribution ($\pm 1 \cdot 10^{-11} m^2/s$) (Table 2.1). LMP has a high molecular weight of approximately several hundred kDa (De Vries, Rombouts, Voragen, & Pilnik, 1982) and is characterized by a broad range of molecular weights. This explains the broad diffusion coefficient distribution exhibited by LMP. On the other hand, WPI has a narrow molecular weight distribution, thus possessing a narrow diffusion coefficient distribution.

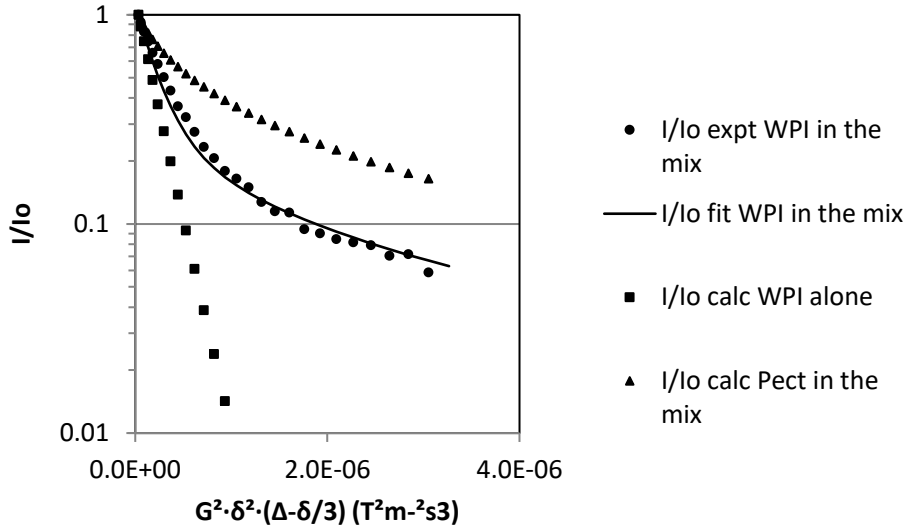


Figure 2.6 Decomposition of protein diffusion signal with pectin (WPI-LMP conjugates ratio 1:1, incubated for 16 days) into the calculated protein diffusion signal without pectin (Lyophilized WPI incubated for 16 days) and the calculated pectin signal with protein pectin (WPI-LMP conjugates ratio 1:1, incubated for 16 days).

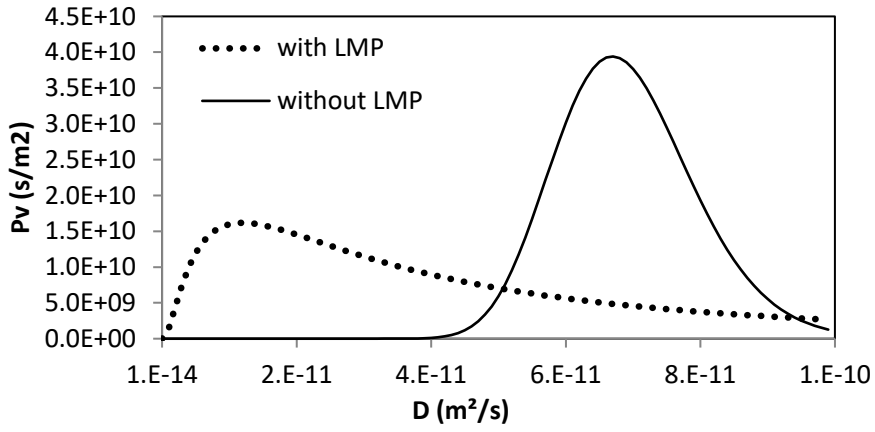


Figure 2.7 Lognormal mass-weighted diffusion coefficient distribution of the protein signals upon dry heat treatment in the absence and presence of LMP.

Upon incubation of WPI and LMP, the interaction of LMP and WPI leads to an increase in molecular weight. Thus a change in the average diffusion coefficient as well as in the distribution width will be observed (Table 2.1). It was noticed that conjugated WPI acquired a similar diffusion behaviour as LMP molecules. On the other hand, the diffusion of conjugated LMP was not significantly affected by the molecular interaction.

Table 2.1 Arithmetic mean diffusion coefficient (D_a) and arithmetic standard deviation (σ_a) value of WPI, LMP, and WPI-LMP conjugates (Day 16) obtained upon fitting Eq. 2.1 to the diffusion signal of the WPI and LMP contributions in the non-conjugated and conjugate samples.

NMR signal (ppm)	WPI		LMP		WPI-LMP	
	D_a (m^2/s)	σ_a (m^2/s)	D_a (m^2/s)	σ_a (m^2/s)	D_a (m^2/s)	σ_a (m^2/s)
0.3-1	$6.9 \cdot 10^{-11}$	$1.0 \cdot 10^{-11}$	-	-	$8.8 \cdot 10^{-11}$	$14.1 \cdot 10^{-11}$
2.5-3.3	$6.8 \cdot 10^{-11}$	$0.9 \cdot 10^{-11}$	-	-	-	-
3.2-4.2	-	-	$6.7 \cdot 10^{-11}$	$28.2 \cdot 10^{-11}$	$5.4 \cdot 10^{-11}$	$15.0 \cdot 10^{-11}$
5.1-5.6	-	-	$3.9 \cdot 10^{-11}$	$9.5 \cdot 10^{-11}$	$3.3 \cdot 10^{-11}$	$8.8 \cdot 10^{-11}$

Whereas it was expected that interaction between two compounds would result in a lower diffusion coefficient, Table 2.1 showed that the arithmetic mean diffusion coefficient of WPI increased slightly to $8.8 \cdot 10^{-11}$ m^2/s upon incubation of WPI and LMP, accompanied by a broader distribution width whose value increased substantially from $1 \cdot 10^{-11} m^2/s$ (narrow) to $14.1 \cdot 10^{-11}$ m^2/s (broad). This phenomenon is due to the fact that the distribution width of WPI in the presence of pectin increased significantly (Fig. 2.7). In fact, for a constant geometric mean, the arithmetic mean increases with increasing distribution width.

From the calculation of the decomposition of the protein signal of the mixture into the WPI signal without pectin and the pectin signal of the mixture according to equation 2.3 (Figure 2.6), it was found that approximately 52 to 59% of the WPI did not react with the LMP. Whereas the former value was obtained from a least squares approach on the measured I/I_o values, the latter was obtained when minimizing the sum of squared differences based on $\ln(I/I_o)$. From the TNBS results, it was found that approximately 15% of the amino groups were lost upon incubation for 16 days. It means that 15% of the primary amino groups in the WPI

were no longer free which could be due to the complex formation through Maillard reaction. On the other hand, the NMR diffusion results indicated that 41 to 48% of the WPI interacted with LMP. The pronounced difference between the TNBS and NMR results follows logically from the fact that whey proteins contain several amino groups per molecule: as an example, β-lactoglobulin, the most abundant whey protein, contains 15 lysine residues. Hence, a major part of the whey proteins can become conjugated to polysaccharides, despite of only a small reduction in the free amino group content.

2.3.2 Heat stability of WPI-LMP Conjugates

The functional properties and structure of proteins can change due to heat treatment. Loss of solubility, structural unfolding, and heat induced aggregation, are some of the consequences from the changed structure of proteins due to heat (Einhorn-Stoll, et al., 2005). Thus, the loss of solubility can be used as an indicator of protein stability against heat. In particular, the loss of solubility of proteins leads to a subsequent loss of their functionality (Kato, 2002).

In this research, the heat stability of protein was evaluated based on the solubility of protein before and after heating. Hereby, a high protein solubility after heat treatment was desired as it can broaden the applications of WPI in food applications. The heat stability experiment was conducted at 80^{o}C since at this temperature WPI undergoes irreversible denaturation.

In Figure 2.8, it can be seen that mixtures of protein and polysaccharides (WPI-LMP conjugate Day 0) at all ratios had a protein solubility of about 90%. Statistically, there was no significant effect of WPI-LMP ratio on the solubility of the protein, while the duration of the incubation period had a significant effect. Furthermore, two-way ANOVA revealed that there was a significant interaction between ratio and incubation day on the solubility of unheated protein. This means that the difference between means of the protein solubility depends on the combination of WPI-LMP ratio and incubation time (Figure 2.8).

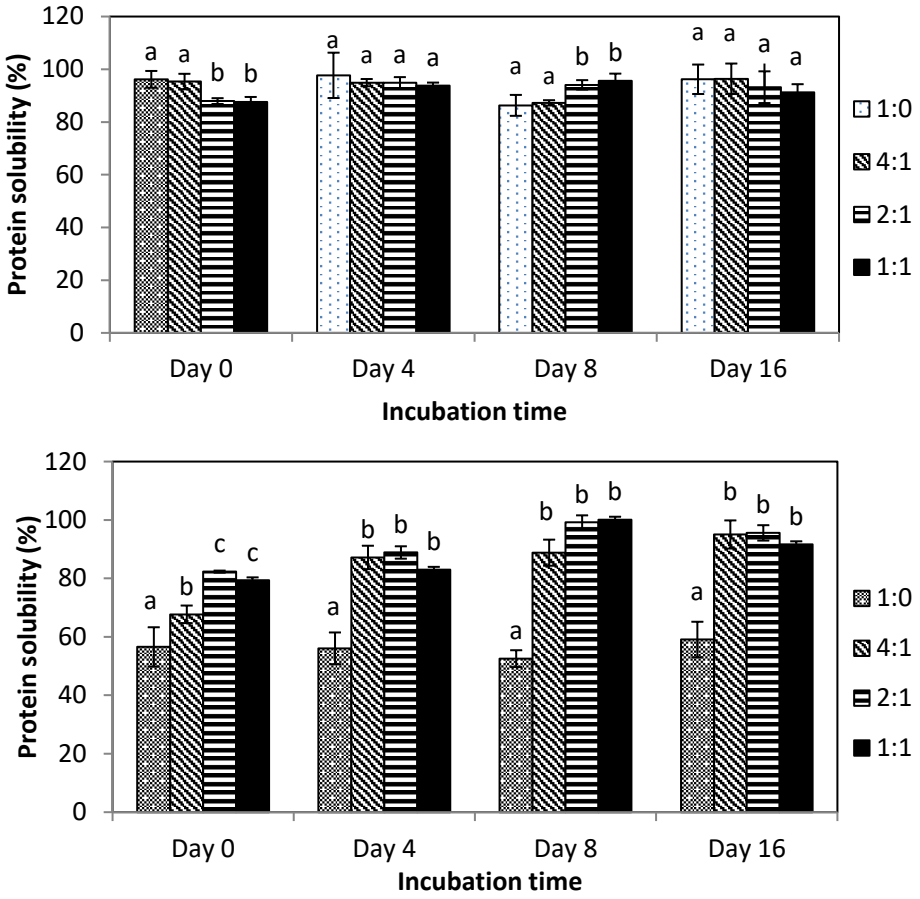


Figure 2.8 Protein solubility (%) and standard deviation bar of WPI alone (Ratio 1:0 Day 0), mixture of WPI-LMP (Day 0), and conjugate of WPI-LMP (Day 4, 8, 16) before (top) and after heat treatment (bottom) at 80^{o}C and pH 6.5 for 2 minutes. Sample with the same letter within the same group (Days) indicates no statistical different between them.

Both pH and temperature are among the factors that have an impact on the solubility of proteins. The solubility of proteins is generally reported to be minimum at its IEP and higher both above and below the IEP because of mutual charge repulsion (Demetriades, et al., 1997a; Pelegrine, et al., 2005). In this experiment, the conjugates were diluted at pH 6.5 in the presence of 30 mM of NaCl. It was reported before that whey protein also had low heat stability at pH values around 6.8 to 7.0, especially in the presence of NaCl (Demetriades, et al., 1997b; Pelegrine, et al., 2005; Ryan, et al., 2012). Thus, it was expected that at this pH the effect of conjugation between WPI and LMP in improving the heat stability of WPI could be observed.

In Figure 2.8, it can be observed that by applying heat, the solubility of the protein was generally reduced. Without addition of pectin, the protein solubility was significantly lower.

Statistical analysis showed that both WPI-LMP ratio and incubation time had a significant influence on the residual solubility of the protein upon heating. There was also a significant interaction between the effect of WPI-LMP ratio and incubation time on the protein solubility after heat treatment (Figure 2.8).

After heat treatment, the protein solubility of WPI-LMP ratio 1:0 at all incubation time points (WPI only) was significantly lower than that of WPI-LMP mixtures and WPI-LMP conjugates. Heating for 2 minutes at 80^{o}C reduced the protein solubility of WPI by almost half. The heat stability of the dry heated WPI did not improve as the incubation time was prolonged. Without incubation, the WPI-LMP ratio 2:1 and 1:1 resulted in a comparable protein solubility and both had a significantly higher protein solubility than that of WPI-LMP ratio 4:1. Whereas the presence of free or weakly complexed polysaccharides (unincubated) was reported to adversely affect the functionality of the protein (Dickinson & Galazka, 1991), a clear beneficial effect of the electrostatic interaction at pH 6.5 is observed in our experiments, which is proportional to the pectin content of the mixtures. The mixture showed to have a better heat stability than the native WPI. This could be due to the protective effect that came from the presence of LMP. Additionally, electrostatic interaction between WPI and LMP might be present in the mixture upon sample preparation which also contributed to the protection of WPI against heat induced aggregation.

When the mixtures were incubated for 4 days, it was found that the residual protein solubility after heating of WPI-LMP conjugates of ratio 4:1, 2:1, and 1:1 was not significantly different. The same trend was obtained for WPI-LMP conjugates incubated for 8 and 16 days. Regarding the incubation time, these results can be linked to the degree of graft reaction of the conjugates. In general, the results showed that conjugates with a higher degree of graft reaction possessed a better heat stability. Therefore, it can be stated that conjugation plays an important role in the improvement of the heat stability of whey proteins. The heat stability analysis results showed that the solubility of the protein could be improved by adding LMP and was further improved upon incubating the mixture of WPI-LMP.

During heat treatment, a change in hydrophobic, electric, and structural properties can generate changes in solubility and functionality of proteins. The mechanism of how conjugates can exhibit heat stability is still being an interesting research topic. The stabilizing

effect of LMP towards heat induced WPI aggregation is suggested to be due to the steric repulsive forces provided by LMP. Conjugation of protein and polysaccharides will combine the surface-active properties coming from the hydrophobic parts of proteins and the steric stabilization properties of the hydrophilic groups coming from the polysaccharides (Dickinson, 1993a). These hydrophilic groups help improving the solubility of WPI. Hereby, conjugates can minimize the exposure of reactive sites of the protein during heat treatment inhibiting interaction between unfolded proteins which can lead to aggregation (Schmitt, et al., 1998; Shu, et al., 1996a). Our results are in agreement with the findings of Jiménez-Castaño, López-Fandiño, et al. (2005) in which β-lactogblobulin which was incubated with dextran at a temperature of 60^{o}C and an a_w of 0.44 for 4 days obtained a better thermal stability, even at its IEP.

As mentioned before, it is possible for polymerization to occur during dry heat incubation of WPI. However, the results of the experiments showed that dry heated WPI did not improve the heat stability of WPI. This means that, even if polymerization of protein occurred during incubation of WPI-LMP, the high stability of WPI against heat observed was certainly due to the formation of WPI-LMP conjugates instead of protein polymers/aggregates.

2.3.3 Emulsifying activity and heat stability

The emulsifying activity of the conjugates (ratio 2:1 Day 8) was compared to that of a mixture of WPI-LMP (Ratio 2:1), native WPI, and dry heated WPI (ratio 1:0 Day 8). The results can be observed in Figure 2.9.

WPI and dry heated WPI stabilized emulsions had a comparable droplet size distribution, characterized by a volume-weighted average diameter of 0.90 and 0.89 μm, respectively. Considering the mixture and the conjugates of WPI-LMP, it was found that combination of LMP with WPI was able to produce smaller particle sizes. Despite of the lower protein content (i.e. 0.50% of WPI in WPI stabilized emulsions versus 0.33% of WPI in WPI-LMP conjugate stabilized emulsions), the smallest droplet size (0.61 μm) was obtained from emulsions stabilized with the conjugates. Therefore, it was confirmed that besides improving the heat stability of the WPI, the conjugation of WPI and LMP also improved the emulsifying activity of the WPI. During the production of the emulsions, it is possible that the WPI-LMP conjugates

could rapidly rearrange on the surface of the oil droplet and cover the surface. Hereby, the hydrophobic groups of the protein are anchored in the oil phase, while the LMP conjugated to the protein provides electrosteric stabilization of the oil droplets. Covalently bound LMP has a better effect on the emulsifying properties of WPI than free LMP (Dickinson & Galazka, 1991). Whereas incubation of WPI in the presence of LMP was seen to improve the emulsifying activity of WPI, incubation in the absence of LMP did not have any impact on the emulsifying activity of WPI.

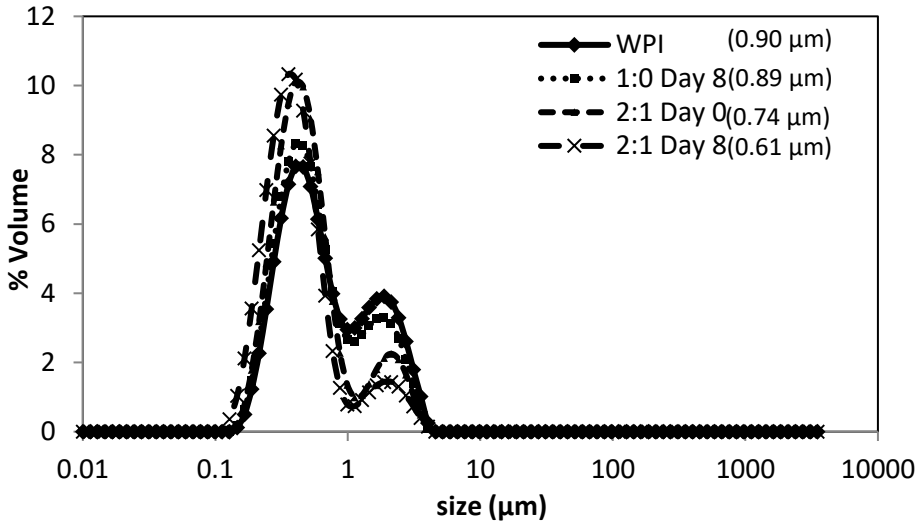


Figure 2.9 Particle size distribution and their respective mass-weighed average diameter ($d_{4,3}$, in µm) of 10% (w/w) o/w emulsions stabilized by 0.5% WPI, dry heated WPI (ratio 1:0 Day 8), mixture of WPI-LMP (ratio 2:1), or WPI-LMP conjugates (ratio 2:1 Day 8) prepared at pH 6.5.

The effectiveness of pectin with a low degree of methyl esterification to improve the emulsifying activity of WPI was recently also reported by Schmidt *et al.* (Schmidt, et al., 2016). The authors compared the emulsifying properties of WPI and citrus pectin conjugates as affected by the degree of esterification of the citrus pectin and revealed that citrus pectin with low degree of methyl esterification gave the highest conjugation yield and smallest droplet size. The finding is in agreement with our result in which the presence of conjugated LMP to WPI improved the emulsifying activity of WPI resulting in a smaller droplet size.

In a last part of the research, the heat stability of the emulsions stabilized with the conjugates was investigated in order to check if the heat stabilizing properties of LMP remain preserved in an O/W emulsion.

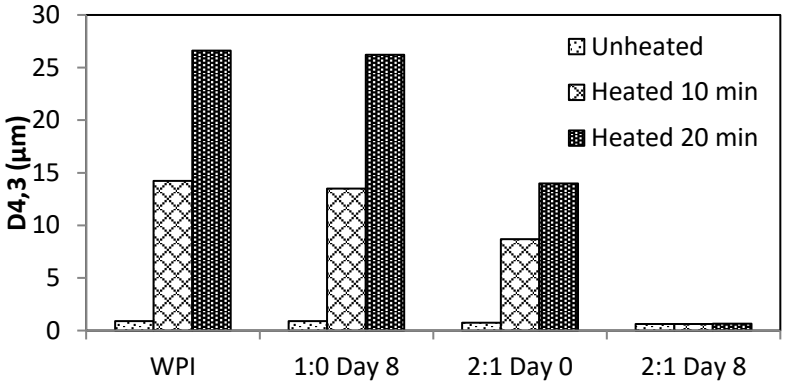


Figure 2.10 Volume-weighted mean diameter ($d_{4.3}$) (µm) of 10% (w/w) o/w emulsions stabilized by 0.5% of WPI, dry heated WPI (8 days), WPI-LMP mixture, or WPI-LMP conjugates of ratio 2:1 (8 days) before and after heating at 80°C for 10 and 20 minutes at pH 6.5.

Upon heating at 80°C for 10 and 20 minutes, emulsions stabilized by WPI and dry heated WPI underwent severe flocculation due to the denaturation and subsequent aggregation of the WPI (Fig. 2.10). In this phenomenon, WPI acts as a glue in between the aggregated droplets (Euston, et al., 2000). Flocculation of the oil droplets was confirmed by measuring the particle size of the heated emulsions with predilution in SDS solution prior to the particle size measurement (data not shown). By using this method, the oil droplet size obtained after 10 minutes of heating became comparable to that of the emulsions before heating. Upon longer heat treatment (20 min) the droplet size obtained using the pre-dilution in SDS solution was still higher than that before heating. This showed that the aggregates could not be completely broken down by dilution in SDS solution or it could be a sign that coalescence occurred in the heated emulsions.

The results included in Fig. 2.10 imply that dry heat treatment of WPI did not improve the heat stability of the WPI stabilized emulsions. Moreover, Fig. 2.10 clearly shows that LMP addition as such was not sufficient to obtain heat stable WPI stabilized O/W emulsion. Only upon dry heat incubation, effective heat stabilization was observed: there was almost no change in the droplet size distribution of WPI-LMP conjugate stabilized emulsions after heating at 80°C. Due

to the fact that WPI, dry heated WPI and WPI-LMP mixtures showed a poor stability against heat, both in solutions and emulsions, it can be concluded that either mixing with LMP (without dry heating) or dry heat treatment (in the absence of LMP) was not sufficient to improve the heat stability of WPI-stabilized emulsions. Hence, these results clearly indicate that the high heat stability exhibited by the emulsions was due to the presence of WPI-LMP conjugates and was not due to the presence of free WPI, polymerized WPI (dry heated WPI) or free pectin. The heat stabilizing activity of the WPI-LMP conjugates was thought to be due to the steric repulsion provided by the LMP attached to the WPI. Upon heating, this steric repulsion is expected to effectively prevent aggregation of thermally unfolded whey proteins, and hence prevent the aggregation of protein-coated emulsion droplets.

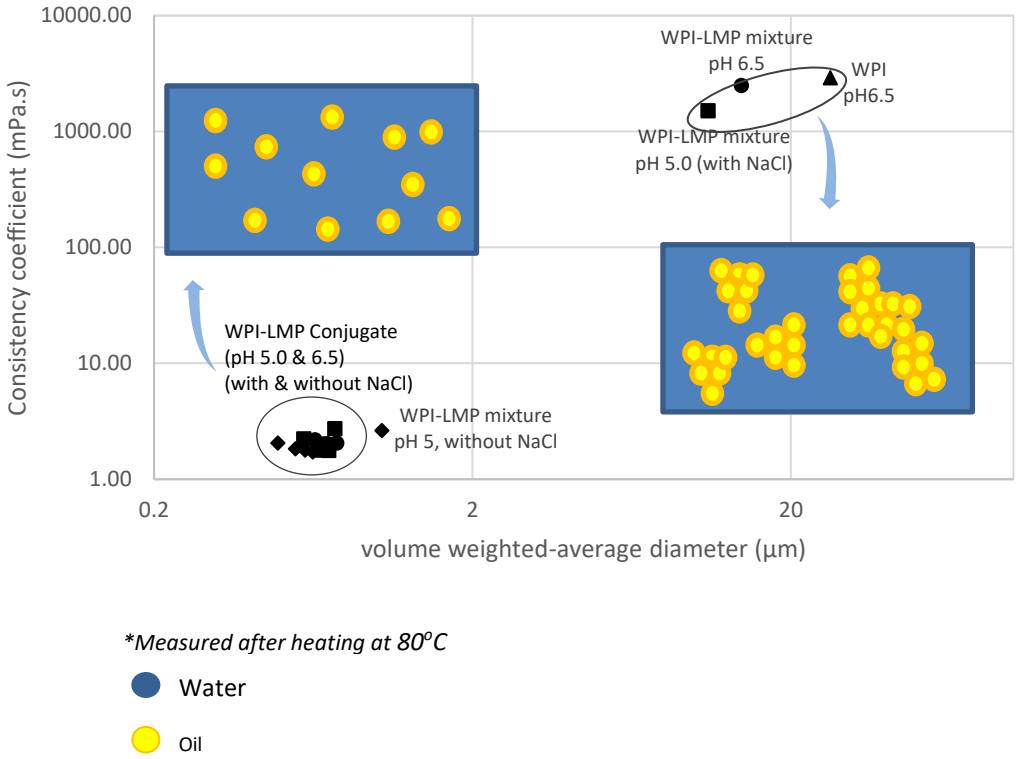
Within the studied time frame (i.e. 4, 8, 16 days) no significant effect of incubation time could be observed. The effect of incubation time (degree of conjugation) on the heat stability of emulsions will be further explored in future research. Anyway, the current results indicate that a shorter dry heat treatment time may be sufficient, which is clearly an important aspect for the possible valorisation of this technology to prepare more heat-stable emulsifiers.

2.4 Conclusion

Upon incubation of WPI and LMP, the Maillard reaction took place resulting in compounds with a higher molecular weight (WPI-LMP conjugates). The conjugates had a better heat stability compared to the native WPI and to mixtures of WPI-LMP. The longer was the incubation time, the higher was the degree of the graft reaction obtained in the conjugates which resulted in a higher heat stability of the WPI. Protein polymerization was observed in the dry heated WPI. Even though it is presumed that polymerization could also take place during incubation of WPI and LMP, the heat stabilizing effect of the dry heat treated mixtures was clearly shown to be due to the presence of conjugates and not to protein polymers. Besides improving the heat stability of WPI, conjugation of WPI and LMP also improved the emulsifying activity of WPI: WPI-LMP conjugates produced smaller oil droplets than native WPI, dry heated WPI, and mixture of WPI-LMP. Moreover, the conjugate strongly increased the heat stability of WPI complexes. Overall, our results indicate that dry heat treatment of protein-pectin mixtures is a promising procedure to improve the protein's functional properties.

CHAPTER 3

IMPROVED HEAT STABILITY OF WHEY PROTEIN ISOLATE STABILIZED EMULSIONS VIA DRY HEAT TREATMENT OF WPI AND LOW METHOXYL PECTIN: EFFECT OF PECTIN CONCENTRATION, PH, AND IONIC STRENGTH



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Abstract

In this study, Whey Protein Isolate (WPI) was conjugated with Low Methoxyl Pectin (LMP) as a means to improve the heat stability of whey protein stabilized oil in water (O/W) emulsions. Hereby, the emulsifying activity and heat stability of the emulsions (10% w/w of oil) stabilized by 0.5% WPI-LMP conjugates were compared to the stability of emulsions stabilized by either 0.5% WPI, 0.5% of a mixture of WPI-LMP, or 0.5% dry heated WPI. The influence of LMP concentration, pH (i.e. 6.5 versus 5.0), and ionic strength (i.e. 0 and 30 mM of NaCl) on the stability of the emulsions was studied. It was found that unheated emulsions stabilized by WPI-LMP conjugates had a relatively small droplet size and were stable against creaming, irrespective of the pH and ionic strength of the emulsions. Results also showed that not only the pH sensitivity, but also the heat-sensitivity of WPI-stabilized emulsions could be significantly improved by application of pectin. Upon heating at 80^{o}C and 120^{o}C up to 20 minutes, there was almost no change in the particle size distribution of the conjugate-stabilized emulsions after heating. Viscosity measurements showed a similar trend; heat treated emulsions stabilized by conjugates did not show any change in consistency. This pronounced heat stabilizing effect of LMP, however, was not observed in emulsions stabilized by a mixture of WPI-LMP. With regard to the dry heat incubation time, a 2 days incubation period was sufficient to produce a WPI-derived emulsifier with superior stability towards both pH and heat treatment.

Keywords: Whey Protein Isolate, Low Methoxyl Pectin, dry heat treatment, heat stability, emulsion, conjugate

3.1 Introduction

Oil in water (O/W) emulsions exist in many food products such as milk, mayonnaise, or vinaigrette. In addition, this type of emulsion is also often prepared to encapsulate some functional compounds such as fat soluble vitamins and β-carotene for health purposes and for drug delivery (Almeida & Souto, 2007; Mao, Dubot, Xiao, & McClements, 2013; Muhoza, Karangwa, Zhang, & Xia, 2016). An emulsifier is required in this system in order to stabilize the emulsion. Nowadays, natural emulsifiers (such as egg yolk, lecithin, and proteins) are mostly preferred (Dickinson, 1993b). According to Garti (1999a) and Dickinson (2011), food grade ingredients such as proteins and polysaccharides are promising natural compounds that can be used to replace synthetic emulsifiers (Dickinson, 2011; Garti, 1999a).

Whey is a byproduct of the milk industry, particularly cheese processing. Based on its high protein content, WPI has been widely used as food ingredient due to its various functionalities (Bryant & McClements, 2000). Thus, it has been known that WPI is a good emulsifier (Damodaran, et al., 1997; Gunasekaran, et al., 2007; Schmitt, et al., 1998), foaming agent (van der Ven, Gruppen, de Bont, & Voragen, 2002), and gelling agent (Gunasekaran, et al., 2007). Nevertheless, the application of WPI as an emulsifier should not only consider the ability of WPI to stabilize the emulsions against destabilization e.g. by flocculation and coalescence during storage (Damodaran, 2005), but also during processing. Many food products are heat treated to prolong their shelf life (Wijayanti, Bansal, Sharma, et al., 2014), which eventually also affects the structure of the emulsions during storage and transportation (Leal-Calderon, Thivilliers, & Schmitt, 2007). Thus, in the application of WPI, it is important that WPI can withstand a heat treatment while at the same time stabilize the emulsions. However, having its denaturation temperature at around 75-80 ^{o}C (Bernal & Jelen, 1985a), WPI is very heat labile and can easily denature. This well-known heat induced denaturation of whey proteins in its turn leads to the alteration of their functional properties (Kessler, et al., 1991), whereby heat treatment of WPI stabilized emulsions may cause protein aggregation and droplet flocculation (Leal-Calderon, et al., 2007), thus altering the characteristics of the emulsions, such as their consistency which may be transformed from liquid to highly viscous fluid and, in the worst case, to a gel, which is undesirable. This limited heat stability is still a major problem for the application of WPI in the food industry (Dissanayake, Ramchandran, Donkor, & Vasiljevic, 2013a).

Previous research has shown that combining the amphiphilic properties of proteins and the hydrophilic properties of polysaccharides can help improving the functional properties, including the heat stability of whey proteins (Jiménez-Castaño, López-Fandiño, et al., 2005; Zhu, Damodaran, & Lucey, 2008). This can be achieved through conjugation of proteins and polysaccharides using the dry heat treatment method (Aoki, et al., 1999; Bu, et al., 2015; Einhorn-Stoll, et al., 2005; Kato, 2002). In this method, a dry mixture of proteins and polysaccharides is exposed to heat at a controlled relative humidity (Kato, 2002). This method is considered to be safe as no chemical additives are needed (Damodaran, 2005; Kato, 2002). Upon incubation, the proteins and polysaccharides will be covalently linked to each other via a Maillard type reaction (Dickinson, 2008).

In this research WPI was covalently linked to Low Methoxyl Pectin (LMP) through dry heat treatment. Pectin was chosen due to its abundant availability which makes it a cheap source of polysaccharides. The performance of WPI-LMP conjugates as emulsifier in O/W liquid emulsions as well as their heat stability were evaluated. The focus of the study was set on the capability of the WPI-LMP conjugates to create stable emulsions against creaming and to protect the emulsions against heat induced denaturation and aggregation. Since the heat stability of WPI stabilized emulsions is influenced by external factors, such as pH and ionic strength, these factors were also taken into consideration in this study. Moreover, different concentrations of LMP in the conjugates, as well as different durations of the incubation time were applied to evaluate their influence on the emulsifying activity of the conjugates and the heat stability of the emulsions stabilized by the conjugates.

3.2 Materials and Methods

3.2.1 Materials

WPI was purchased from Davisco Foods International Inc. (Le Sueur, MN, USA). Protein analysis revealed that the WPI contains approximately 97.7% protein, whereby 85% of the protein is β-lactoglobulin (Van der Meeren, et al., 2005). Low Methoxyl Pectin (LMP) (Unipectin OB700) was obtained from Cargill (Ghent, Belgium) and contained 89.6% of dry matter. The LMP was used without further purification. Oil in water emulsions were prepared using sunflower oil purchased from a local supermarket.

3.2.2 Conjugates preparation

Conjugates were prepared through dry heat treatment at controlled relative humidity. The conjugates were prepared from a 5% (w/v) protein solution and 1% (w/v) LMP solution. Correction on the protein content and dry matter content of the WPI and LMP, respectively, was performed when preparing the solutions. The solutions were prepared in distilled water and the pH of the solutions was adjusted to 7.0 with 1.0 N HCl to avoid formation of ionic complexes that might form at lower pH during mixing (Mishra, Mann, & Joshi, 2001). Subsequently, the solutions were kept at refrigerator temperature overnight before mixing. Solutions were mixed at a WPI to LMP ratio of 1:0, 4:1, 2:1 and 1:1 (on weight basis) and were frozen afterwards. The concentration of WPI was kept constant at all mixing ratios, while the concentration of pectin was varied.

The frozen samples were lyophilized (Alpha 1-2 LD plus, Christ) to remove all the water and obtain a dry product. The freeze dried products were then dry heat treated by incubation at a temperature of 60^{o}C for up to 16 days in a desiccator containing a saturated NaCl solution to keep the relative humidity at ± 74% (Greenspan, 1977).

3.2.3 Emulsion preparation

To investigate the influence of dry heat incubation time on the emulsifying activity of the conjugates and the heat stability of the emulsions, emulsions stabilized by a WPI-LMP mixture (ratio 2:1, day 0) and WPI-LMP conjugates incubated at different incubation times (ratio 2:1, day 1-16) were prepared. These emulsions were compared to emulsions stabilized by native WPI (WPI), freeze dried WPI (ratio 1:0, day 0) and dry heated WPI (ratio 1:0, day 4-16) as controls. The emulsions were prepared in imidazole buffer and sodium acetate buffer at both pH 6.5 and pH 5.0, respectively, to investigate the influence of pH. Furthermore, at pH 5.0, the emulsions were prepared in the absence and presence of 30 mM NaCl to study the effect of ionic strength on the heat stability of protein-stabilized emulsions around the protein's isoelectric point. Emulsions stabilized by WPI-LMP mixtures and conjugates at different LMP concentration (WPI:LMP ratio 4:1, 2:1, and 1:1) were prepared to study the influence of LMP concentration on the emulsifying activity of the WPI-LMP mixtures and conjugates as well as on the heat stability of the emulsions.

Initially, the aqueous phase containing 0.5% of WPI or 0.5% WPI-LMP mixture or conjugates was prepared. The aqueous phase was then kept overnight in the fridge prior to emulsification to fully hydrate the hydrocolloids. An appropriate amount of oil was added to the aqueous phase to have 10% (w/w) of oil in the final emulsion. The mixture was premixed using an IKA Ultra-turrax TV45 (Janke Kunkel, Staufen, Germany) at the highest speed (24000 rpm) for 1 minute. Subsequently, the premix was homogenized using a Microfluidizer 110S for 2 minutes at 4 bar of compressed air pressure corresponding to 560 bar of liquid pressure. The heat exchanger coil of the Microfluidizer was immersed in a water bath set at 55^{o}C during emulsification.

3.2.4 Heat coagulation test

The heat coagulation test was conducted based on the method developed by Kasinos, Karbakhsh, and Van der Meeren (2015). The test was performed at both 80^{o}C and 120^{o}C in an oil bath. To ensure a homogenous temperature distribution in the oil bath, an IKA RW20 stirrer with a 3 bladed metallic propeller set at 250 rpm was put in the corner of the oil bath. The temperature of the oil bath was monitored using a thermometer and a thermocouple connected to an electronic digital thermometer (Agilent 34970A, Diegem, Belgium).

Approximately 9 mL of emulsion was brought into 20 mL headspace vials (75.5 x 22.5 mm, 1 st hydrolytic class, Grace, Deerfields, IL, USA) and sealed tightly with a metallic cap. The emulsions were then placed in a metallic rack and immersed in the oil bath. Heating of the emulsions was carried out for 20 minutes. Afterwards, the emulsions were cooled and kept at room temperature before further measurement. All the measurements were performed within one day after the emulsion preparation.

3.2.5 Particle size analysis

The particle size distribution analysis was performed using a Mastersizer 3000 (Malvern Instrument Ltd, Malvern, UK) equipped with red and blue light source. The refractive index used was 1.47, while the absorbance index was set at 0.01. The sample was added dropwise to the wet sample dispersion unit (Malvern Hydro MV) until an obscuration level between 10-20% was obtained. To avoid multiple scattering effects, the emulsions were prediluted 10 times in distilled water before the measurement. The stirrer of the dispersion unit was set at

1500 rpm during measurement; a higher stirring intensity was avoided to prevent disintegration of aggregates.

3.2.6 Microscopy observation

The oil droplets within the emulsions were observed using a CX40 light microscope (Olympus GmbH, Hamburg, Germany) equipped with an Axiocam ERc5s camera (ZEISS, Germany). The observation was done at a magnification of 100X with addition of immersion oil to improve the image quality obtained from the microscope.

3.2.7 Viscosity

A LV-DVII+pro portable viscometer (Brookfield) was employed to measure the viscosity of the emulsions. The measurements were performed at a temperature of 20°C at different shear rates. Spindle SC4-18 and SC4-34 were used to measure the viscosity of samples with a liquid consistency and with high viscosity (i.e. paste and gel like consistency), respectively. To obtain the shear rates (in 1/s), the speed of rotation (in rpm) was multiplied with a conversion factor, which was 1.32 and 0.28 for spindle SC4-18 and SC4-34, respectively.

For emulsions with liquid consistency, 8 mL of sample was needed to fill the sample holder, whereas for emulsions with a gel-like consistency, the measurement was performed in the container of the emulsion. The apparent viscosity data obtained were fitted to a power law equation as can be seen in Equations 3.1, 3.2, and 3.3 whereby τ represents the shear stress (in Pa), C the consistency coefficient (in Pa.s), and $\dot{\gamma}$ the shear rate (in s^{-1}). Parameter n represents the flow behaviour index, in which n=1 represents Newtonian flow, n<1 represents pseudoplastic behaviour, and n>1 represents dilatant behaviour.

$$\tau = C \times \dot{\gamma}^n \quad \text{(Equation 3.1)}$$

As $\tau = \mu \times \dot{\gamma}$ (Equation 3.2)

Equation 3.2 can be rewritten as $\mu = C \times \dot{\gamma}^{(n-1)}$ (Equation 3.3)

In this study, the viscosity of emulsions with Newtonian behaviour (n ≈ 1) was obtained from the average value of the viscosities measured at different shear rates. In the case of samples which exhibited shear thinning behaviour, the consistency coefficient was obtained by fitting the power law equation to the experimental data.

3.2.8 Accelerated creaming stability evaluation

The creaming stability of the emulsions was evaluated using a LUMiFuge 116 (LUM GmbH, Germany). In this method, 0.4 mL of the prepared emulsion was filled into a rectangular polycarbonate cell, with a depth of 2.2 mm and a width of 8.0 mm, and exposed to a centrifugation force of 1200 g. The test was conducted for 60 minutes during which the device recorded the light transmission through the sample as a function of sample height in the test tube every 60 seconds. Front tracking data analysis was applied to the raw data within the region from 96.5 to 112.5 mm from the center of rotation by setting the trigger value at 20% transmission. Using this method, a curve which shows the position of the interface between serum and cream phase during centrifugation was obtained. The slope of this curve represents the creaming velocity of the emulsions at 1200 g.

3.2.9 Electrophoretic mobility

The electrophoretic mobility of the emulsions stabilized by WPI, WPI-LMP mixtures, and WPI-LMP conjugates was measured in 20 mM of Na-acetate buffer at different pH values using a Zetasizer 2c (Malvern Ltd, UK). The emulsions were diluted 1000x in the buffers and let to stand overnight prior to the measurement.

3.2.10 Protein Load

The emulsions were centrifuged using a Beckman L7-55 ultraspeed centrifuge with SW 40 Ti rotor (Pasadena, CA, USA) at 40000 g and 10^{o}C to separate the oil phase (cream) from the aqueous phase. Initially, the emulsions were loaded into Beckman polyallomer centrifuge tubes (13 x 15 mm, Beckman Coulter, Pasadena, CA, USA) and centrifuged for 105 minutes. Afterwards, the cream layer was removed while the serum and the sediment phase (if any) were mixed and stored for protein load determination. The protein content of the serum and aqueous phase was determined by Kjeldahl analysis and the results were calculated using a conversion factor of 6.38. From this value, the protein recovery, i.e. the ratio between the amount of protein found in the serum and the total protein content of the aqueous phase before emulsification, was determined.

The protein load of the emulsions can be calculated using the equations below:

$$\text{Protein Load} = \frac{(1-\text{protein recovery}) * C_{wpi} * C_{pro}}{\text{SSA} * \text{C oil}} \quad \text{(Equation 3.4)}$$

$$\text{SSA (specific surface area)} = \frac{6}{d_{3.2} * \rho} \ (\text{m}^2/\text{kg}) \quad \text{(Equation 3.5)}$$

$$\text{Protein recovery} = \frac{\%\ \text{protein in serum}}{\%\ \text{protein in aqueous phase before emulsification}} \quad \text{(Equation 3.6)}$$

In (3.4), C_{wpi} is the WPI concentration in the emulsions, which was 0.5% (w/w) for WPI-stabilized emulsions and 0.33% (w/w) for WPI-LMP conjugate stabilized emulsions, C_{pro} is the protein content of the WPI (i.e. 92.6%), C_{oil} is the oil fraction in the emulsion (i.e. 10% (w/w)), and SSA is the specific surface area of the oil droplets in the emulsions (in m^2/kg oil). The latter calculation should be performed using the Sauter diameter ($d_{3.2}$) of the non-aggregated oil droplets, i.e. before heating. Lastly, ρ is the oil density (in kg/m^3)

3.2.11 Statistical Analysis

The statistical analysis was generated using SPSS 22 (IBM). One-way Anova was performed on the results of protein load analysis at a significance level of 95%. A paired sample *t*-test was conducted at a significance level of 95% to compare the creaming velocity of the emulsions stabilized by WPI-LMP conjugates (dry heat treated for 2-16 days) at pH 5.0 and 6.5, as well as before and after heating at 80 and 120 ^oC.

3.3 Results and Discussion

This study evaluated both the emulsifying activity of WPI-LMP conjugates and the heat stability of the emulsions stabilized by the WPI-LMP conjugates at pH 5.0 and 6.5, which were selected to create conditions in the close vicinity and away from the WPI's isoelectric point, respectively.

3.3.1 Particle size of emulsions

The particle size of the emulsions was measured to evaluate the emulsifying activity of WPI-LMP conjugates and the heat stability of the emulsions. Generally at pH 6.5, WPI was able to form submicron emulsions, both with and without conjugation (Fig. 3.1). Nevertheless, the emulsions underwent severe heat aggregation upon heating as shown in Fig. 3.1. Microscopic images revealed that strong droplet aggregation occurred in the WPI stabilized emulsions

after heating for 20 minutes (Fig. 3.2); even dilution with 1% SDS prior to the droplet size measurement could not break the aggregates (data not shown). As SDS should be able to break aggregates by eliminating bridging flocculation (Roesch, Rincon, & Corredig, 2004) and hydrophobic interactions (Floury, Desrumaux, & Legrand, 2002), the resistance to SDS showed that the aggregates in the heated WPI stabilized emulsions were formed through irreversible protein aggregation, which may involve the formation of disulphide bridges between the free thiol groups on whey proteins (Vasbinder, et al., 2003).

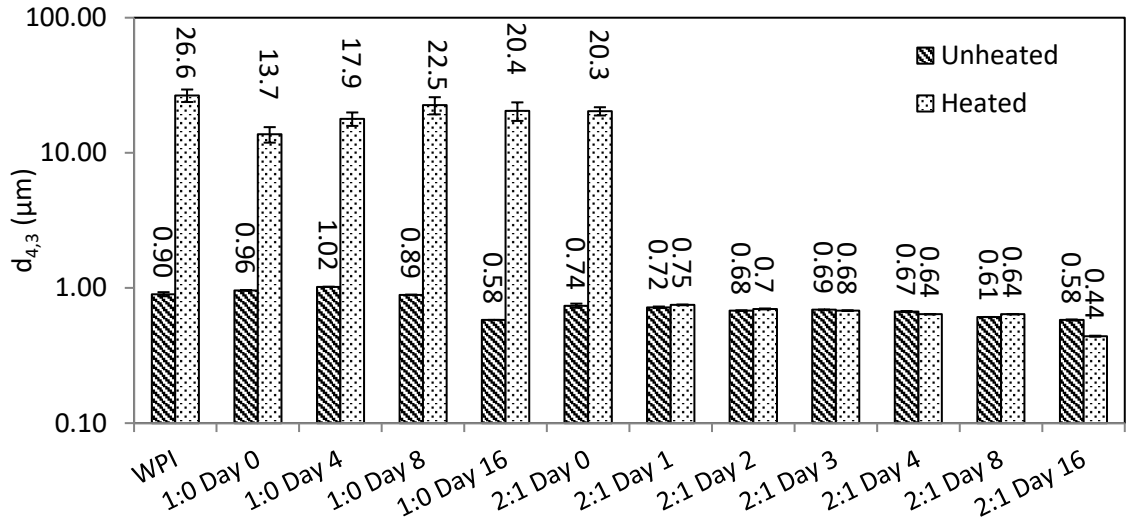


Figure 3.1. Volume-weighted average oil droplet size ($d_{4.3}$) (with standard deviation) of the 10% (w/w) o/w emulsions stabilized by 0.5% of native WPI, freeze dried WPI (WPI:LMP ratio 1:0, Day 0), dry heated WPI (WPI:LMP ratio 1:0, Day 4-16), WPI-LMP mixture (WPI:LMP ratio 2:1, Day 0) and WPI-LMP conjugates (WPI:LMP ratio 2:1, Day 1-16) prepared at pH 6.5, before and after heating at 80^{o}C for 20 minutes.

In the presence of LMP at a WPI:LMP ratio of 2:1, WPI was also able to produce submicron emulsions, regardless of the incubation time. Furthermore, the emulsions produced were finer than in the absence of LMP. Nevertheless, regarding the heat stability, the emulsions were unstable towards heating without a preliminary dry heat incubation of both biopolymers. Therefore, to obtain a heat stable emulsion, both biopolymers should be conjugated. Fig.3.1 clearly shows that a minimum of 1 day incubation was required to have heat stable emulsions.

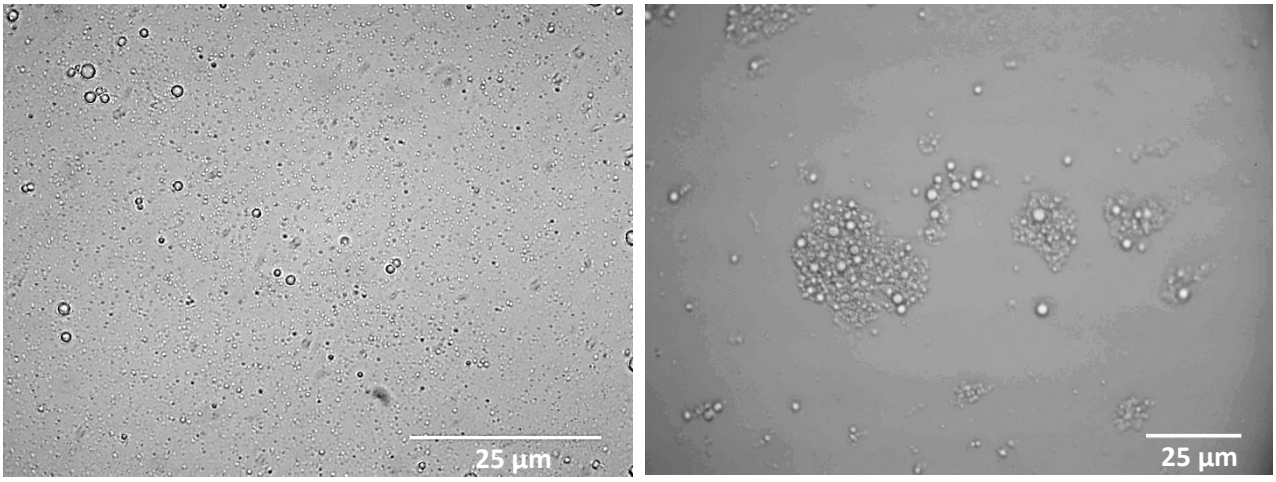


Figure 3.2. Microscopic image of a 10% (w/w) o/w emulsion stbailied by 0.5% WPI (pH 6.5) before (left) and after (right) heating at 80^{o}C for 20 min, when diluted (10x) in distilled water.

The stabilization in WPI stabilized emulsions is attributed to the presence of electrostatic repulsive forces, which is highly influenced by the pH of the system. Above and below the isoelectric point (IEP) of WPI, the droplets of a WPI stabilized emulsion have a positive or negative net charge, respectively, which stabilizes the emulsions against droplets aggregation (Demetriades, et al., 1997a). On the other hand, at pH conditions around the IEP of WPI, the droplets have zero net charge (Demetriades, et al., 1997a) and thus droplet aggregation is expected. Around its IEP, surface denaturation of the attached protein can lead to droplet flocculation due to hydrophobic interaction and disulphide bond formation between the oil droplets (McClements, 2004) leading to formation of droplet aggregates. This phenomenon was observed in the emulsions stabilized by WPI and dry heated WPI at pH 5.0 (data not shown). Heating of the emulsions resulted in even more pronounced droplet aggregation. This phenomenon occurred both in the absence and presence of NaCl, with more pronounced droplet aggregation in the latter case.

Table 3.1. Volume-weighted average droplet diameter ($d_{4.3}$) of 10% (w/w) o/w emulsions stabilized by 0.5% of a WPI-LMP mixture (WPI:LMP ratio 2:1, 0 days incubation) and WPI-LMP conjugates (WPI:LMP ratio 2:1, 1-4 days incubation) prepared at pH 5.0 in the absence and presence of 30 mM NaCl before and after heating at 80°C and 120°C for 20 minutes.

NaCl (mM)	Incubation time (days)	$d_{4,3}$ (µm)		
		Before	After (80 °C)	After (120 °C)
0	0	0.93 ± 0.01a	1.04 ± 0.01	-
	1	0.75 ± 0.00b	0.63 ± 0.00	-
	2	0.60 ± 0.00c	0.60 ± 0.00	-
	3	0.63 ± 0.00d	0.56 ± 0.00	-
	4	0.56 ± 0.00e	0.49 ± 0.00	-
30	0	1.03 ± 0.01a	9.64 ± 1.24	85.90 ± 4.12
	1	0.76 ± 0.00b	0.74 ± 0.00	0.74 ± 0.00
	2	0.59 ± 0.00c	0.67 ± 0.00	0.60 ± 0.00
	3	0.60 ± 0.00c	0.71 ± 0.00	0.55 ± 0.00
	4	0.59 ± 0.00c	0.59 ± 0.00	0.52 ± 0.00

In the presence of LMP, without conjugation, and in the absence of NaCl, the droplet size of the emulsions was slightly larger at pH 5.0 than at pH 6.5 and the emulsion was heat stable (Table 3.1). At pH 5.0, it is expected that WPI formed an electrostatic complex with LMP. This complex might be responsible for the surface activity of the mixture as well as the stabilization of the emulsions against heat. Nevertheless, in the presence of 30 mM NaCl, the same result was not obtained (Table 3.1): the volume-weighted average droplet size largely increased upon heating. Upon conjugation of WPI and LMP, the droplet size of the emulsions was comparable to that at pH 6.5. Further dry heat incubation of the WPI-LMP mixture for 8 and 16 days was found to produce emulsions with an average droplet size of 0.45, and 0.49 µm, respectively. This shows that the average droplet size of the emulsions was getting smaller as the dry heat incubation time was extended, although the difference was not remarkable. It was previously reported that conjugation of WPI with maltopentose did not affect the emulsifying activity of WPI (Li, Enomoto, Ohki, Ohtomo, & Aoki, 2005). However, our results showed that conjugation of WPI with LMP through dry heat treatment slightly improved the emulsifying activity of WPI. Furthermore, a previous study also revealed that the droplet size of emulsions stabilized by WPI and citrus pectin conjugates was influenced by the yield or degree of conjugation between WPI and pectin (Schmidt, et al., 2016). The improved

emulsifying activity of the WPI-LMP conjugates could be attributed to the presence of an additional steric force provided by the conjugated LMP present at the surface of the oil droplets as well as to better adsorption of the conjugates to the surface of the oil droplets during emulsification due to an increase in the hydrophobicity of WPI caused by protein unfolding during glycosylation (Akhtar, et al., 2007; Kato, Tsutsui, Matsudomi, Kobayashi, & Nakai, 1981; Zhu, et al., 2010). The performance of the conjugates to create emulsions with a small droplet size is an important factor since the particle size has a great influence on the stability of emulsions against creaming (Huang, Kakuda, & Cui, 2001).

As far as the effect of heating on the droplet size of the emulsions is concerned, Table 3.1 clearly shows that no effect of heating and NaCl was observed in the emulsions stabilized by conjugated WPI-LMP after heating the emulsions at 80 and 120^{o}C. Since the LMP was covalently linked to the WPI, WPI acted like an aid to bring the LMP on the surface of oil droplets. This polysaccharide then contributed to the stability of the emulsions by creating a thick steric barrier which protected the oil droplets (Zhu, et al., 2010). This barrier also provided an additional steric force to prevent droplets to come in close proximity during heating which prevented their heat induced aggregation. Aside from that, the presence of covalently linked polysaccharides to proteins also improves the hydrophilicity of oil droplets which also contributes to the stabilization of the emulsion (Akhtar, et al., 2007; Diftis, et al., 2006b; Zhu, et al., 2010).

Table 3.2. Volume-weighted average oil droplet size ($d_{4.3}$) of 10% (w/w) o/w emulsions stabilized by 0.5% of WPI-LMP mixtures (0 days incubation) and WPI-LMP conjugates (8 days incubation) at different WPI:LMP ratios prepared at pH 5.0 in the presence of 30 mM NaCl before and after heating at 80^{o}C for 20 minutes.

WPI:LMP	Incubation time (days)	d4,3 (µm)	
		Before	After
4:1	0	22.10 ± 1.90	24.60 ± 0.30
2:1		1.03 ± 0.00	9.64 ± 1.20
1:1		0.99 ± 0.00	0.86 ± 0.00
4:1	8	0.75 ± 0.00	0.67 ± 0.00
2:1		0.55 ± 0.00	0.56 ± 0.00
1:1		0.46 ± 0.00	0.44 ± 0.00

In the presence of NaCl, a higher concentration of LMP was apparently required for the WPI-LMP mixture to stabilize the emulsions at pH 5.0. Table 3.2 depicts that a mixture with WPI-LMP ratio of 1:1 could stabilize the emulsion at pH 5.0 in the presence of NaCl. However, this was not achieved at lower LMP concentration (ratio 4:1). In contrast, in the case of conjugates, lowering the LMP concentration by increasing the WPI-LMP ratio to 4:1 gave a comparable result as obtained at higher LMP concentration. Table 3.2 also indicates that WPI-LMP conjugates created emulsions with a smaller droplet size than mixtures of WPI-LMP. As the concentration of the LMP was increased, there was a slight decrease of the droplet size of the emulsions stabilized by the WPI-LMP conjugates. Nonetheless, the difference was not significant. This phenomenon could be related to the degree of conjugation: as more LMP is present in the conjugate, the degree of conjugation is higher, which seems to improve the emulsifying activity of the conjugates (Setiowati, et al., 2016).

The dry heat treatment incubation time seemed to have an impact in the presence of LMP. Without incubation (Day 0), the WPI-LMP mixture was found to be less surface active (Table 3.1 and 3.2). It was expected that the emulsions stabilized by WPI-LMP conjugates incubated for longer times would have a better stability against heat. However, the results in this study showed that there was no difference in the heat stability of the emulsions stabilized by WPI-LMP conjugates incubated for 1, 2, 3, 4, 8, and 16 days: all these emulsions maintained their droplet size after heating. Hence, 1 day of dry heat conjugation was already sufficient to prevent heat induced droplet aggregation. This phenomenon could be due to the fact that all of these conjugates had a relatively comparable degree of conjugation, which according to (Setiowati, et al., 2016) ranged from 15 to 19%. On the other hand, dry heat treatment of WPI in the absence of LMP did not have any influence on the emulsifying activity of WPI and on the heat stability of the emulsions.

3.3.2 Viscosity of emulsions

Droplet aggregation in whey protein stabilized emulsions changes not only the droplet size but also the viscosity of the emulsion (Sliwinski, et al., 2003). According to Drapala, et al. (2016a), heating of protein stabilized emulsions induces protein denaturation and aggregation leading to an increase in their viscosity. Thus, a change in viscosity after heating can be an indicator of emulsion heat instability.

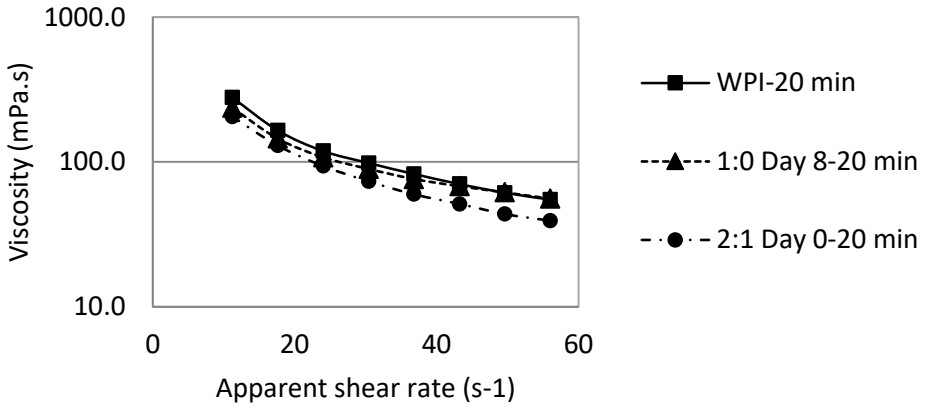


Figure 3.3. Viscosity profile of 10% (w/w) o/w emulsions stabilized by 0.5% native WPI, dry heated WPI (WPI:LMP ratio 1:0, Day 8), and WPI-LMP mixture (WPI:LMP ratio 2:1, Day 0) prepared at pH 6.5 after heating at 80°C for 20 minutes.

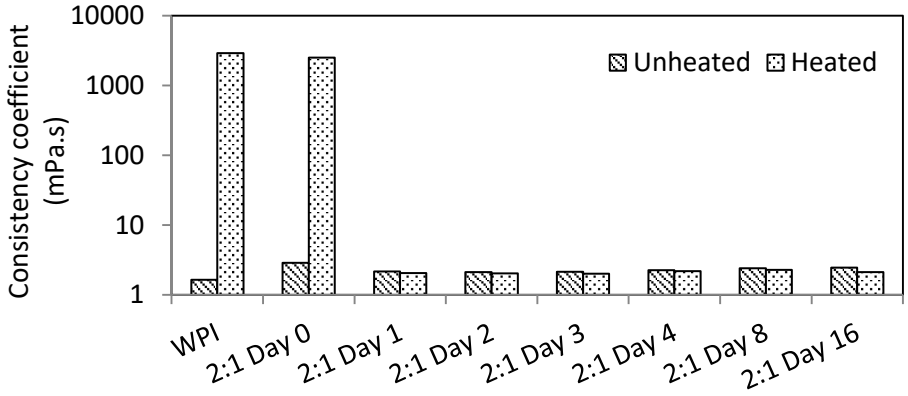


Figure 3.4. Consistency coefficients of the 10% (w/w) o/w emulsions stabilized by 0.5% of native WPI, WPI-LMP mixture (WPI:LMP ratio 2:1, Day 0) and WPI-LMP conjugates (WPI:LMP ratio 2:1, Day 1-16) before and after heating at 80°C for 20 minutes, prepared at pH 6.5.

At pH 6.5, all emulsions behaved as Newtonian fluids. Whereas emulsions stabilized by WPI and dry heated WPI had a viscosity of approximately 1.7 mPa.s, in the presence of LMP, the viscosity of the emulsions increased to 2-3 mPa.s with the highest viscosity obtained for the emulsion stabilized by a mixture of WPI-LMP (without incubation). Heating generally led to increase in viscosity, which was shown to be strongly shear rate dependent if no conjugates are present, i.e. in the presence of native WPI, dry heated WPI or WPI-LMP mixtures (Fig. 3.3). Hence, neither dry heat treatment nor pectin addition is effective on its own; instead, conjugation by dry heat treatment in the presence of pectin is needed (Fig. 3.4). Figure 3.4 also reveals that 1 day of conjugation was already sufficient to stabilize the emulsions towards heat treatment. Previous studies also reported that the viscosity of whey protein stabilized

emulsions increased after heating due to the presence of aggregates, whereby conjugation of whey proteins with maltodextrin could prevent this phenomenon (Drapala, et al., 2016a).

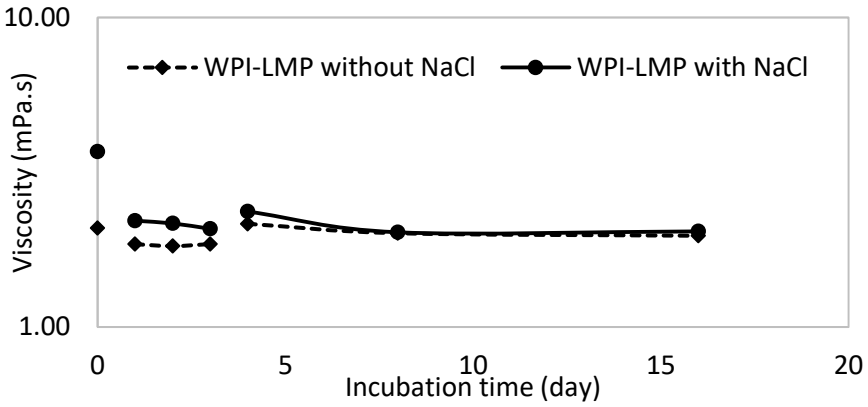


Figure 3.5. Viscosity profile of unheated 10% (w/w) o/w emulsions stabilized by 0.5% WPI-LMP conjugates (ratio 2:1) prepared at pH 5.0, without and with 30 mM NaCl.

At pH 5.0, in the absence and presence of NaCl, the unheated emulsions stabilized by WPI exhibited a distinctly higher viscosity than emulsions stabilized by WPI in the presence of LMP due to the presence of aggregates in the emulsions. In the absence of NaCl, emulsions stabilized by a WPI-LMP mixture had a comparable viscosity with WPI-LMP conjugates stabilized emulsions. However, by adding 30 mM of NaCl, the viscosity of the emulsions stabilized by a WPI-LMP mixture became higher than that of emulsions stabilized by WPI-LMP conjugates (Fig. 3.5) indicating the presence of aggregates (Xu, Wang, Jiang, Yuan, & Gao, 2012). On the other hand, the viscosity of unheated emulsions stabilized by WPI-LMP conjugates prepared by dry heating for 1, 2, 3, 4, 8, and 16 days were comparable at pH 5.0 in the absence and presence of 30 mM NaCl. In Fig. 3.5, the viscosity of emulsions stabilized by WPI-LMP conjugates which were dry heated for 1 to 3 days was observed to be lower than that of emulsions stabilized by WPI-LMP conjugates which were dry heated for 4 to 16 days; this difference might be due to the fact that the former conjugates were prepared from a different batch of LMP. Hence, the viscosity of emulsions stabilized by WPI-LMP conjugates which were dry heated during 1-3 days is shown as a different series. Nonetheless, it still can be observed that there was no noticeable influence of incubation time on the viscosity of unheated emulsions stabilized by WPI-LMP conjugates, obtained by dry heat treatment for 1-16 days. This again points to effective prevention of pH-induced droplet aggregation by dry heat conjugates.

Upon heating for 20 minutes, the emulsions stabilized by WPI and dry heated WPI prepared at pH 5.0 underwent transformation into a gel-like structure accompanied with a prominent increase in their viscosity. Furthermore, syneresis and phase separation was also observed, which is in line with the observations reported by Drapala et al. (2016a). Hence, it was not possible to measure the viscosity of these emulsions. In the absence of NaCl, no effect of heating was observed in the emulsions stabilized by a mixture of WPI and LMP (Table 3.3). In contrast, in the presence of NaCl, limited aggregation before heating and extended aggregation upon heating was observed. However, this was prevented by conjugation of the WPI-LMP mixture. Table 3.3 shows that emulsions prepared with WPI-LMP conjugates which were prepared by dry heating for 1, 2, 3, and 4 days exhibited an excellent stability towards heat at pH 5.0 in the absence and presence of 30 mM of NaCl. It was also discovered that heating the emulsions at sterilization temperature (120^{o}C) did not increase the viscosity of the emulsions. This is an interesting finding since the test was performed at a pH around the IEP of the protein and in the presence of 30 mM of NaCl. Similar to the results of droplet size measurement, there was no noticeable effect of extending the incubation time up to 16 days on the viscosity of the emulsions.

Table 3.3. Consistency coefficients (in mPa.s) of the 10% (w/w) o/w emulsions stabilized by 0.5% of a WPI-LMP mixture (0 days incubation) or WPI-LMP conjugates (1-4 days incubation) at a WPI:LMP ratio of 2:1 prepared at pH 5.0 in the absence and presence of 30 mM NaCl.

NaCl (mM)	Incubation time (days)	consistency coefficient (mPa.s)		
		Before	After (80^{o}C)	After (120^{o}C)
0	0	2.09	2.64	-
	1	1.85	1.72	-
	2	1.83	1.79	-
	3	1.85	1.84	-
	4	2.15	2.05	-
30	0	3.69	1512	-
	1	2.20	2.75	1.90
	2	2.16	1.79	1.88
	3	2.08	1.78	1.82
	4	2.36	2.23	1.87

It should be noted that increasing the concentration of LMP in the mixture of WPI-LMP to an equal concentration of WPI (ratio of 1:1) enabled the mixture to prevent an increase in the

viscosity of the emulsions after heating at pH 5.0 in the presence of NaCl (Fig. 3.6). This result was not achieved at a lower concentration of LMP (e.g. ratio 2:1 and 4:1), which indicates the importance of a sufficiently high LMP concentration in WPI-LMP mixtures. On the other hand, stable emulsions were obtained both before and after heating using WPI-LMP conjugates, even at low concentrations of LMP (i.e WPI:LMP ratio 4:1) (Fig. 3.6).

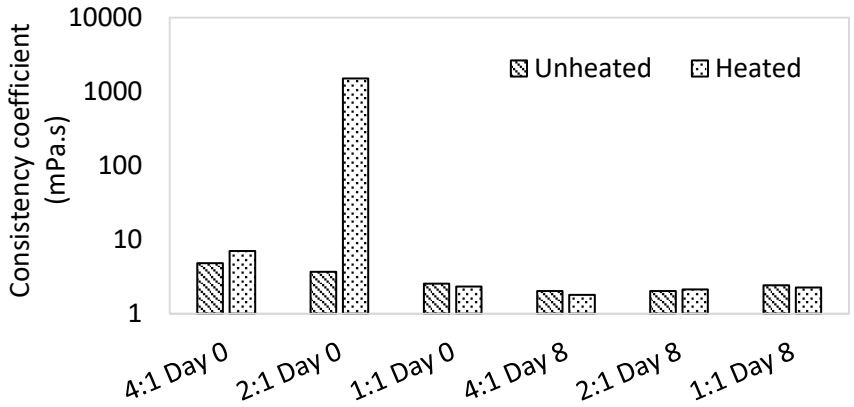


Figure 3.6. Consistency coefficient of 10% (w/w) o/w emulsions stabilized by 0.5% WPI-LMP mixtures (Day 0) and conjugates (Day 8) at different WPI:LMP ratios prepared at pH 5.0 in the presence of 30 mM NaCl, before and after heating at 80°C for 20 minutes.

3.3.3 Emulsion stability against creaming

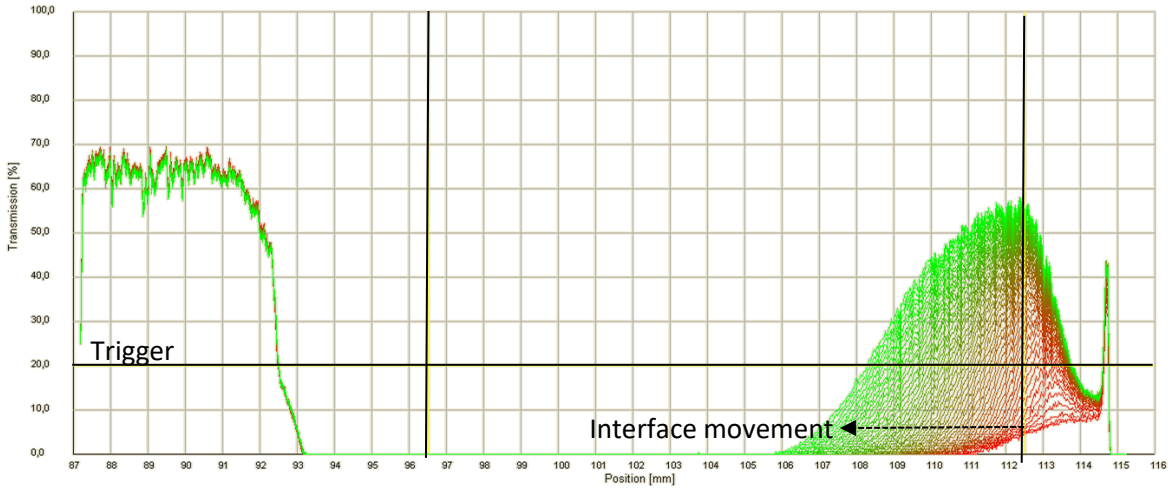


Figure 3.7. Typical analytical centrifugation profile of a 10% (w/w) o/w emulsion containing 0.5% WPI-LMP conjugates (with WPI:LMP ratio 2:1) dry heat treated for 16 days at pH 5.0 in the presence of 30 mM NaCl measured at 1200 g for 1 hour.

The stability of the emulsions against creaming during storage was evaluated using analytical centrifugation. A typical profile can be seen in Fig. 3.7. During centrifugation, the position of the interface in the emulsions was recorded from which a plot of interface location versus time could be developed. The slope obtained from this curve was equal to the creaming velocity at 1200 g. The measurements were performed on both unheated and heated emulsions to evaluate the emulsion stability against creaming as influenced by heating. However, emulsions with a gel like consistency could not be analysed using this method; this was the case for all heated WPI-stabilized emulsions without any LMP, which were heavily aggregated.

Table 3.4 shows the creaming velocity of emulsions prepared at pH 6.5 and pH 5.0 in the absence and presence of NaCl. In this study, the emulsions stabilized by dry heat treated WPI prepared at pH 5.0 underwent extensive protein aggregation even before heating. Furthermore phase separation was observed within an hour after the emulsion was prepared and therefore the creaming velocity measurement of the emulsions was not performed. Since the emulsions stabilized by WPI and dry heated WPI were not heat stable at 80^{o}C, the heat stability test of the emulsions at 120^{o}C was not conducted. The heat stability of emulsions stabilized by WPI-LMP conjugates which were dry heated for 16 days was not tested at 120^{o}C since 8 days of dry heat treatment already resulted in emulsions with excellent heat stability.

Comparing the creaming velocity of the emulsions at pH 6.5 and at pH 5.0 in the absence and presence of NaCl, a paired t-test indicated that the emulsions had a significantly lower creaming velocity at pH 6.5 than at pH 5.0 ($p<0.05$). This was expected as WPI stabilized emulsions are not very stable around their IEP. Additionally, it could be observed that a longer incubation time for conjugate preparation resulted in a lower creaming velocity, which is in line with the smaller droplet sizes as described in Figure 3.1 (for pH 6.5) and Table 3.1 (for pH 5.0).

Table 3.4. Creaming velocity of 10% (w/w) o/w emulsions (in mm/day) (mean ± standard deviation) stabilized by 0.5% of native WPI, freeze dried WPI (WPI:LMP ratio 1:0, Day 0), dry heated WPI (WPI:LMP ratio 1:0, Day 4-16), WPI-LMP mixture (WPI:LMP ratio 2:1, Day 0) or WPI-LMP conjugates (WPI:LMP ratio 2:1, Day 1-16) at 1200 g at pH 6.5 and 5.0 in the absence and presence of 30 mM NaCl before and after heating for 20 minutes

Emulsion	pH 6.5		pH 5.0		pH 5.0, 30mM NaCl		
	unheated	heated 80°C	unheated	heated 80°C	unheated	heated 80°C	heated 120°C
WPI	164 ± 1	*	566 ± 228	*	536 ± 101	*	*
1:0 Day 0	160 ± 5	*	*	*	-	-	-
1:0 Day 4	158 ± 8	*	*	*	-	-	-
1:0 Day 8	148 ± 6	*	*	*	-	-	-
1:0 Day 16	126 ± 15	*	*	*	-	-	-
2:1 Day 0	268 ± 0	*	240 ± 0	288 ± 0	568 ± 1	*	*
2:1 Day 1	116 ± 0	139 ± 1	209 ± 1	207 ± 0	166 ± 1	171 ± 0	443 ± 3
2:1 Day 2	128 ± 1	126 ± 1	165 ± 1	188 ± 1	196 ± 1	179 ± 1	222 ± 0
2:1 Day 3	105 ± 1	113 ± 1	158 ± 1	178 ± 1	191 ± 1	192 ± 0	206 ± 1
2:1 Day 4	88 ± 9	84 ± 7	126 ± 2	126 ± 1	144 ± 0	142 ± 1	196 ± 1
2:1 Day 8	80 ± 3	79 ± 1	129 ± 1	126 ± 1	143 ± 0	141 ± 4	190 ± 1
2:1 Day 16	57 ± 1	62 ± 2	128 ± 0	127 ± 1	128 ± 0	123 ± 2	-

**: Not determined due to its gel-like consistency;* *- : experiment was not performed*

At pH 6.5, the emulsions stabilized by WPI-LMP conjugates exhibited a superior creaming stability as compared to those stabilized by WPI, dry heated WPI, and mixtures of WPI-LMP (Table 3.4). Despite having a smaller droplet size than WPI stabilized emulsions, unheated emulsions stabilized by mixtures of WPI-LMP exhibited a lower creaming stability than WPI stabilized emulsions. It has to be noted that despite the fact that the droplet size of the emulsions was smaller, the viscosity of the emulsions was higher due to the presence of free (unconjugated) LMP. This might induce (either depletion or bridging) flocculation in the emulsions, which may lead to creaming. It was found that only emulsions stabilized by the conjugates could retain their low creaming velocity after heating. According to Dickinson (1992), O/W emulsions with micron-sized oil droplets may be considered to be stable when the creaming velocity in the gravitational field is below 1 mm/day (i.e. at 1 g). Considering the fact that the creaming velocity values presented in Table 3.4 were determined in a centrifugal field at 1200 g, it follows that all emulsions prepared with the WPI-LMP conjugates may be considered to be stable towards creaming in the gravitational field.

The pH was found to have more influence on the emulsions stabilized by mixtures of WPI-LMP. At a pH where electrostatic interactions between WPI and LMP may occur (i.e. at pH 5.0), the mixture, without conjugation, is sufficient to ensure the stability of the emulsions before and after heating: at pH 5.0 in the absence of NaCl, adding LMP as such was enough to protect the emulsion from pH- and heat-induced droplet aggregation. Nevertheless, the emulsion underwent creaming twice faster than WPI-LMP conjugates stabilized emulsions. This could be due to the fact that the droplet size of the emulsion was bigger than that stabilized by the conjugates which based on Stokes' law will lead to a higher creaming velocity (Dickinson, 1992). Furthermore, bridging flocculation induced by electrostatic interaction of proteins and polysaccharides in which polysaccharide molecules may simultaneously interact with proteins attached at two or more different droplets (Singh, Tamehana, Hemar, & Munro, 2003) could also accelerate the creaming of the emulsion. On the other hand, LMP addition could not prevent heat destabilization at pH 6.5, where the electrostatic interactions counteracted protein-pectin interaction.

A different phenomenon was observed when 30 mM NaCl was added at pH 5.0: a higher concentration of LMP in the mixture of WPI-LMP was required to stabilize the emulsions (Table 3.5). Whereas a low amount of LMP (ratio 4:1) in the mixture of WPI-LMP resulted in an emulsion with poor stability, the same amount was sufficient to produce emulsions with excellent stability when the mixture was conjugated. Regardless of the LMP concentration, when the mixture of WPI and LMP was conjugated, the creaming velocity of the emulsions decreased considerably. Furthermore, the creaming stability of the emulsions stabilized by the WPI-LMP conjugates was hardly influenced by the presence of NaCl (Table 3.4). Upon heating at a higher temperature (120^{o}C), the emulsions stabilized by the conjugates exhibited a slight increase in their creaming velocity (Table 3.4). Whereas droplet size measurement showed no noticeable change in the droplet size of the emulsion stabilized by conjugates which were incubated for 1 day, creaming velocity measurement showed a marked increase after heating. By incubating WPI-LMP mixtures for 2 days or more, the stability towards creaming was hardly affected by heating. The paired sample t test revealed that the creaming velocity of emulsions stabilized by WPI-LMP conjugates which were dry heated for 2 to 16 days before and after heating at both 80 and 120^{o}C were not significantly different ($p>0.05$).

Table 3.5. Creaming velocity (in mm/day) (mean ± standard deviation) at 1200 g of 10% (w/w) o/w emulsions stabilized by 0.5% of WPI-LMP mixtures (0 day incubation) or WPI-LMP conjugates (8 days incubation) at different WPI:LMP ratios prepared at pH 5.0 in the presence of 30 mM NaCl before and after heating at 80^{o}C for 20 minutes.

WPI:LMP	Incubation time (days)	Creaming Velocity (mm/day)	
		Unheated	Heated
4:1	0	1467 ± 180	3867 ± 395
2:1		567.8 ± 1.0	*
1:1		313.3 ± 0.7	310.1 ± 2.0
4:1	8	173.8 ± 1.4	170.3 ± 2.3
2:1		143.1 ± 0.1	141.0 ± 4.5
1:1		126.7 ± 0.1	127.5 ± 0.7

**: Not determined due to its gel-like consistency.*

Overall, the results shown in Table 3.4 and 3.5 indicate that WPI-LMP conjugates could be applied within a wider range of pH than electrostatic complexes. This will broaden the application of WPI as it is not limited by the pH of the products where it is added into. In contrast, WPI-LMP electrostatic complexes are unstable at neutral pH and require a stabilization means such as by heating the mixture at a temperature above the denaturation temperature of WPI in order to stabilize the complex at neutral pH (Gentès, et al., 2010). Besides being less affected by the pH, emulsions stabilized by conjugates of WPI-LMP were also not influenced by the presence of low concentrations of NaCl.

3.3.4 Electrophoretic Mobility of emulsions

The EM measurement showed that the emulsions stabilized by WPI had a neutral charge at around pH 5.0 which is widely known as the IEP of WPI (Demetriades, et al., 1997a). At this pH, WPI-stabilized emulsions do not have enough repulsive forces to prevent droplet aggregation; thus, unstable emulsions are obtained. The further the pH of the emulsions was removed from the IEP of WPI, the bigger the EM of the WPI-stabilized emulsion became, and thus the more stable the emulsion was expected. Comparing the EM of emulsions stabilized by WPI and WPI-LMP conjugates at different ratio (4:1, 2:1, and 1:1) revealed that the presence of LMP shifted the EM of the emulsions to a more negative value. This is owing to the fact that pectin carries a negative charge. This phenomenon also indicates that the LMP was adsorbed at the surface of the oil droplets (Neirynck, Van der Meeren, Bayarri Gorbe,

Dierckx, & Dewettinck, 2004; Salminen & Weiss, 2014). At pH 5.0, in the presence of LMP, the EM value of the emulsions was strongly shifted downwards to a more negative value (Fig. 3.8). Moreover, the isoelectric point of the emulsions stabilized by the WPI-LMP conjugates was also found to be shifted to a lower pH value. Figure 3.8 revealed that WPI-LMP conjugate (ratio 4:1) stabilized emulsions had an IEP at a pH around 3.6. Increasing the concentration of LMP further decreased the IEP value. These observations explained the stability of the emulsions stabilized by WPI-LMP conjugates at pH 5.0. Besides electrostatic stabilization, the droplets were also stabilized through steric stabilization obtained from the presence of LMP at their surfaces.

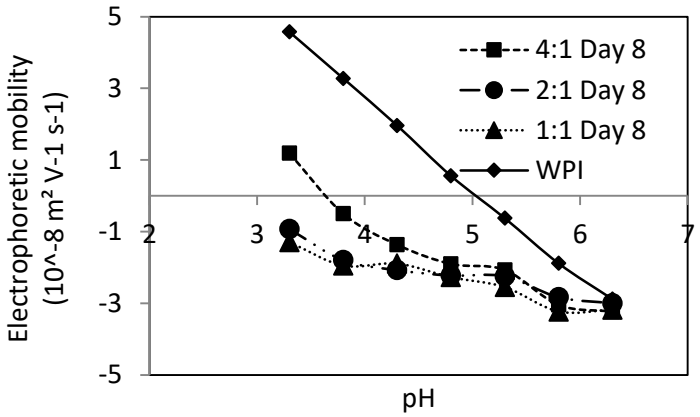


Figure 3.8. Electrophoretic mobility profile of emulsions stabilized by native WPI and by WPI-LMP conjugates with a WPI:LMP ratio of 4:1, 2:1 and 1:1 which were incubated for 8 days.

Regarding the influence of incubation time on the EM of the emulsions, there was no obvious difference between the EM profiles of the emulsions stabilized by WPI-LMP conjugates prepared at different incubation times (i.e. 0, 1, 2, 3, 4, 8, and 16 days of incubation) (data not shown). The emulsions exhibited a comparable EM profile within the pH range of 3.3 to 5.5. At pH 5.0, the electrophoretic mobility of emulsions stabilized by the WPI-LMP mixture (Day 0) was comparable with that stabilized by WPI-LMP conjugates. This showed that there was electrostatic interaction between WPI and LMP and thus LMP was present on the surface of the oil droplets. This explains the stability against creaming and heat induced droplet aggregation of emulsions stabilized by the WPI-LMP mixture at pH 5.0.

At a pH higher than 5.5, it was observed that there was a small influence of incubation time on the EM of the emulsions whereby the emulsions stabilized by the WPI-LMP mixture had a

slightly lower EM than those stabilized by the WPI-LMP conjugates (data not shown). This could mean that less LMP was present on the surface of the oil droplets.

3.3.5 Protein coverage

Table 3.6. Protein load (in mg/m^2) (mean ± standard deviation) of 10% (w/w) o/w emulsions stabilized by 0.5% of WPI, a WPI-LMP mixture (WPI:LMP ratio 2:1, Day 0) or WPI-LMP conjugates (WPI:LMP ratio 2:1, Day 8) before and after heating at 80^{o}C for 20 minutes.

pH	Sample	Unheated	heated
pH 6.5	WPI	$2.32 \pm 0.24^{a,A}$	$3.22 \pm 0.02^{a,B}$
	2:1 Day 0	$1.18 \pm 0.31^{b,A}$	$1.70 \pm 0.26^{b,A}$
	2:1 Day 8	$1.53 \pm 0.32^{b,A}$	$1.52 \pm 0.19^{b,A}$
pH 5.0 (+30 mM NaCl)	WPI	$(36.30 \pm 3.26)^{a,A}$	$(42.90 \pm 0.46)^{a,A}$
	2:1 Day 0	$2.24 \pm 0.10^{b,A}$	$2.75 \pm 0.10^{b,B}$
	2:1 Day 8	$1.46 \pm 0.23^{b,A}$	$1.63 \pm 0.18^{c,A}$

^{a,b} Means in the same column followed by different lowercase letters are significantly different ($p<0.05$).
^{A,B} Means in the same row followed by different uppercase letters are significantly different ($p<0.05$).

Table 3.6 shows the protein load of emulsions stabilized by WPI, a WPI-LMP mixture, and WPI-LMP conjugates. It has to be noted that the WPI-stabilized emulsions contained 0.5% of WPI, while in the presence of LMP, the emulsions contained only 0.33% of WPI. In Table 3.6, it can be observed that a higher concentration of WPI led to more adsorption of protein. The results show that the protein load of unheated emulsions stabilized by the WPI-LMP mixture and by WPI-LMP conjugates was not significantly different at both pH values. However, upon heating at pH 5.0 in the presence of NaCl, the protein load of the emulsion stabilized by a mixture of WPI-LMP increased and became significantly higher than that of the emulsion stabilized by the WPI-LMP conjugates. In contrast, there was no significant increase in the protein load of the emulsions stabilized by WPI-LMP conjugates after heating at both pH values.

Table 3.6 reveals that at pH 5.0 and in the presence of 30 mM NaCl, the protein load of WPI stabilized emulsions was extremely high. Microscopic analysis revealed that the emulsion was unstable and had a high amount of aggregates even before heating (data not shown). Due to this droplet aggregation, the sauter mean diameter ($d_{3,2}$) of the emulsion droplets was

overestimated, and hence (according to equation 3.5) the specific surface area of the oil droplets was underestimated. Since the protein load is inversely proportional to the specific surface area, as can be seen in equation 3.4, it follows that the protein load of these aggregated emulsions was overestimated.

The results indicated that heating caused the protein load of emulsion stabilized by WPI only to increase. It has been reported previously that heating of WPI-stabilized emulsions causes more protein adsorption to the interface of oil droplets, which can promote droplet aggregation (Sliwinski, et al., 2003). Unadsorbed protein is suggested to play an important role in heat induced aggregation of WPI-stabilized emulsions; the aggregation of this type of emulsion has the same kinetics as aggregation of heated whey protein (Sliwinski, et al., 2003). During heating, the unadsorbed proteins unfold and subsequently, aggregation takes place between the unadsorbed proteins and/or between unadsorbed proteins and adsorbed proteins (Kim, Decker, & McClements, 2002). Due to this phenomenon, proteins accumulate on the surface of oil droplets. Similar to this, the unfolded protein in the surface of the oil droplet can also form aggregates with the adsorbed protein within the same droplet or between droplets (McClements, 2004). These protein aggregates will then act as a glue in between the oil droplets leading to the formation of oil droplet aggregates (Euston, et al., 2000). As indicated by the results shown in Table 3.6, heat-induced additional protein deposition was prevented in the presence of conjugated WPI-LMP. It was suggested that in the presence of covalently linked LMP, aggregation of the unfolded protein was prevented due to electrostatic and steric repulsion provided by the LMP and thus droplet aggregation was avoided. Besides additional steric stabilization provided by the covalently linked LMP, stabilization of the emulsions against heat by the conjugates could also be due to a shift on the denaturation temperature of the WPI. It was previously reported that glycosylated WPI has a higher denaturation temperature than native WPI (Liu, et al., 2013).

3.4 Conclusions

Overall, the results indicated that conjugation of WPI and LMP by dry heat treatment could largely improve the functional properties of the protein, particularly the emulsifying activity and heat stability. From this study, several conclusions can be drawn. First of all, the nature of the interaction between WPI and LMP is important; adding LMP to WPI as such was not

sufficient to protect WPI stabilized emulsions from heat induced droplet aggregation in most cases. In addition, the emulsifying activity of the WPI-LMP conjugates with constant WPI to LMP ratio was improved as the incubation time was prolonged, as observed by the decrease in average emulsion oil droplet diameter. On the other hand, the incubation time had hardly any effect on the heat stabilizing properties (within the studied interval), whereby 1 or 2 days of dry heat incubation of a dry WPI-LMP mixture was sufficient to ensure heat stable emulsions. Moreover, the influence of LMP concentration was less pronounced in WPI-LMP conjugates than in WPI-LMP mixtures. Overall, WPI-LMP conjugates stabilized emulsions showed a superior stability against creaming and heat regardless of the pH and ionic strength.

Regarding the future application of WPI-LMP conjugates, it is highly possible to use WPI-LMP conjugates for different applications which are based on an O/W emulsions system. In milk related industries, these conjugates can be applied in the production of concentrated milk and infant milk (Drapala, Auty, Mulvihill, & O'Mahony, 2016b) to prevent protein aggregation during pasteurization and sterilization which normally leads to fouling on the heating surfaces, hereby reducing the heat transfer efficiency. It is also possible to use the conjugates for encapsulation of active compounds both in single oil in water emulsions or in double emulsions (Dickinson, 2011; Lutz, Aserin, Wicker, & Garti, 2009). Moreover, WPI-LMP conjugates have the advantage that they can be applied within a wider range of pH values than mixtures or electrostatic complexes of proteins and polysaccharides. Since WPI-LMP conjugates have a lower IEP than WPI, application around the IEP of WPI is also possible. Moreover, heat sensitivity is a major drawback for WPI application (Dissanayake, et al., 2013a). As conjugation of WPI and LMP has been found to improve the emulsifying activity and especially the heat stability of the stabilized emulsions, this research may largely broaden the application of WPI as an effective O/W emulsifier.

CHAPTER 4

QUARTZ CRYSTAL MICROBALANCE EQUIPPED WITH DISSIPATION (QCM-D) AS A TOOL TO STUDY THE INTERACTION OF WHEY PROTEIN ISOLATE AND LOW METHOXYL PECTIN

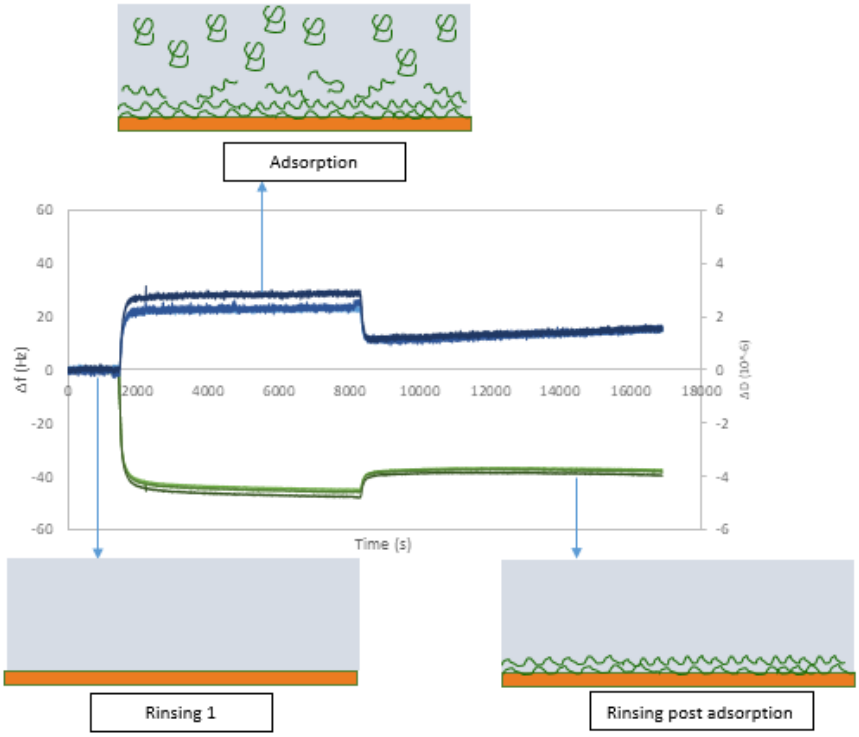


Figure: Typical whey protein adsorption profile on the surface of a hydrophobically modified gold sensor recorded with a QCM-D

Abstract

This study focused on the interaction between WPI and LMP. The adsorption behaviour of WPI at a hydrophobic surface was studied using QCM-D. Information on thickness of the layer and the surface load were able to be extracted from QCM-D. Furthermore, the results also gave insight on the viscoelasticity of the layer as well as on its adsorption kinetics. The result revealed that the WPI layer formed on the surface of the sensor was rather viscoelastic at around neutral pH and more rigid at pH around its IEP. A multilayer was formed upon adsorption of WPI. Interaction of WPI and LMP at around IEP has caused the thickness of the layer and surface load to increase due to electrostatic interaction of WPI and LMP. Furthermore, the layer became much more viscoelastic which was suggested to improve the steric stabilization in the emulsion. WPI-LMP conjugates produced a thick and viscoelastic layer. However identification of the compound present on the surface of the sensor was difficult. Moreover, quantification of the degree interaction between WPI and LMP was not possible. Despite the drawbacks, some essential information on the properties of the film can be drawn from the result of the QCM-D measurement.

4.1 Introduction

Proteins are widely used in food applications due to their various functionalities. Due to their amphiphilic character, whey proteins can offer excellent stability to emulsions (Kinsella, et al., 1989; Lefèvre, et al., 2003). Nevertheless, due to some drawbacks, such as heat lability and pH sensitivity, whey proteins are often combined with other molecules. There have been a lot of studies on combining whey proteins and polysaccharides, for instance pectin, to improve the functionality of whey proteins. Whey protein and pectin can be combined through electrostatic interaction or by conjugation via covalent bonds resulting in whey proteins with improved emulsifying activity (Neirynck, et al., 2004; Salminen, et al., 2014; Setiowati, et al., 2016). Upon emulsification, the hydrophobic groups of whey proteins will anchor at the oil part while the hydrophilic part, provided by the polysaccharides, will stay in the aqueous phase, thus improving the stability of the oil droplets (Dickinson, et al., 1992; Diftis, et al., 2006b). The presence of polysaccharides provides an additional layer at the surface of the oil droplets preventing the droplets to come in close vicinity which can lead to phase separation (Zhu, et al., 2010).

To understand the mechanism of protein and polysaccharide interaction in improving the functionality of whey protein, it is important to study the interaction between these two biopolymers and the phenomena that occur on the surface of oil droplets upon adsorption of these biopolymers. In addition, it is also important to determine the degree of the interaction between the biopolymers. There are a lot of available methods that have been used to quantify the interaction between whey protein isolate and polysaccharides. Most of them involve chemical analysis which sometimes can be labour intensive and time consuming. In the case of whey protein-polysaccharide conjugates, the degree of interaction is normally quantified by measuring the reduction of free amino groups after the conjugation through chemical analysis using reagents such as TNBS (*2,4,6*-trinitrobenzene sulfonic acid) (Jimenez-Castano, et al., 2007; Setiowati, et al., 2016) and OPA (*o*-phthalaldehyde) (Wooster & Augustin, 2006). Additionally, the protein or polysaccharide load on the surface of the oil droplets can also be used to determine the degree of complexation as the polysaccharides will be present on the surface of oil droplets only when they are bound to the proteins while the unbound polysaccharides will remain in the aqueous phase. However, this method is also labour intensive.

In this research, Quartz Crystal Microbalance with Dissipation (QCM-D) equipped with a high gradient probe were proposed as tools to study the interaction between proteins and polysaccharides. QCM-D has gained popularity as a means to study molecular adsorption to a solid surface (Höök, et al., 2001). QCM-D is equipped with a quartz crystal which oscillates at its fundamental resonant frequency (5 MHz for gold sensor) when an electric current is applied. When the applied current is removed, the decay of the oscillation is recorded (Kontturi, Tammelin, Johansson, & Stenius, 2008). The frequency and energy dissipation of the oscillation are influenced by adsorption of the compound (Liu & Kim, 2009). Figure 4.1 shows the influence of a rigid and a viscoelastic layer on the frequency and dissipation recorded by the QCM-D. As adsorption takes place, the resonant frequency decreases accompanied with or without an increase of the energy dissipation depending on the viscoelasticity of the formed layer. A viscoelastic layer tends to dissipate more energy than the rigid one. The QCM-D has several advantages. It is simple and sensitive enough to detect a change of thickness and adsorbed mass (surface load) on the surface of the sensor. Moreover, besides real time observation of the adsorption behaviour, a change in the 1viscoelasticity of the layer can also be monitored, and the viscoelasticity of the layer can be estimated (Österberg, Stenius, & Laine, 2007). The device accommodates the study of both rigid and viscoelastic layers.

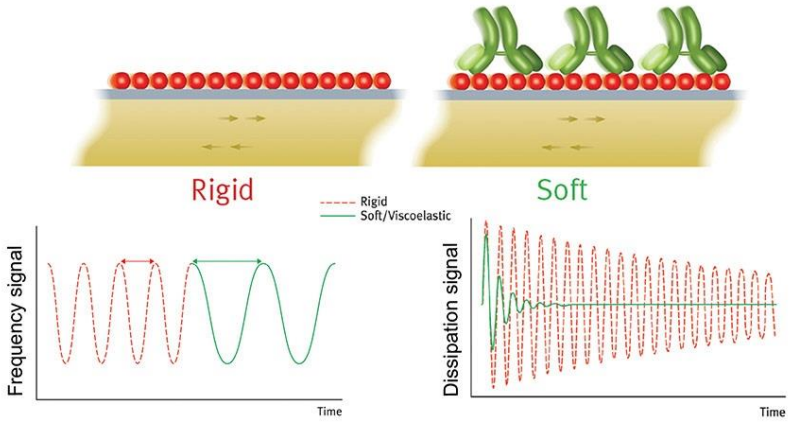


Figure 4.1 Influence of a rigid and a soft (viscoelastic) layer on the frequency and dissipation recorded by QCM-D (image obtained from www.biolinscientific.com)

4.2 Material and Methods

4.2.1 Material

The WPI was purchased from Davisco Foods International Inc. (Le Sueur, MN, USA). Protein analysis revealed that the WPI contained approximately 97.7% protein, whereby 85% of the protein is β-lactoglobulin (Van der Meeren, et al., 2005). The low methoxyl pectin (LMP) (Unipectin OB700) was obtained from Cargill (Ghent, Belgium) and contained 89.6% of dry matter.

4.2.2 Conjugate preparation

A WPI-LMP mixture and WPI-LMP conjugates were prepared following the method described in section 2.2.2.

4.2.3 QCM-D experiments

4.2.3.1 Preparation of the hydrophobic gold sensor

Gold sensors with fundamental frequency of 4.95 MHz were used in these experiments. The gold sensors were cleaned and coated to have a hydrophobic surface (self-assembled monolayer). The sensors were first placed in a UV/ozone chamber for 10 minutes and soaked in piranha solution which consists of Milli-Q water, 25% ammonia, and 30% hydrogen peroxide mixed at a ratio of 5:1:1 at 75 ^{o}C for 10 min. Subsequently, the sensors were rinsed with Milli-Q water and dried with dry air. The dried sensors were then placed in the UV/ozone chamber again for another 10 minutes.

The self-assembled monolayer was formed by soaking the gold sensors in 2mM 1-hexadecanethiol (95%) in ethanol overnight or no less than 16 h (Mivehi et al., 2013; Lebec et al., 2013). The sensors were rinsed thoroughly with ethanol followed by drying using dry air. The contact angle of the coated sensors was then determined.

4.2.3.2 Solution preparation

WPI and LMP were dissolved in 20 mM sodium phosphate buffer or 20 mM of Na-acetate buffer (in Milli-Q water) at different pH (4.5, 5.0, 5.5, 6.0, 7.0) at concentrations of 0.5% and

0.33% (w/w), respectively. Mixtures of WPI-LMP (WPI-LMP Day 0) and WPI-LMP conjugates (ratio 2:1, Day 8) were dissolved in the same buffer at pH 6.55 and 7.0 at a concentration of 0.5%. Sodium phosphate buffers and Na-acetate buffer were used to prepare solutions with a pH of above 6 and below 6, respectively.

4.2.3.3 QCM-D experiments

The QCM-D was cleaned with 2% soap (Hellmanex II) solution and rinsed with Milli-Q water. The Milli-Q water was let to run overnight with the pump speed set at 0.1 mL/min. The experiment was started by pumping buffer solution into the QCM-D chamber until a stable baseline was obtained. Subsequently, the sample solution was pumped to the chamber and let to flow until a stable baseline was observed which indicated that no further adsorption took place. This was then followed by rinsing using the same buffer to remove the loosely adsorbed molecules and to ensure that the same density and viscosity were obtained as during baseline registration. The experiments were performed at 22^{o}C and at a pump speed of 0.1 mL/min.

4.2.3.4 QCM-D data analysis

Data analysis was performed using QTools software 30.15.553 (Biolin Scientific). The Sauerbrey formula was used to determine the mass of the adsorbed protein when the film adsorbed at the surface of the sensor was rigid. On the other hand, when there was overtone dependency or the layer built on the surface of the sensor was viscoelastic, the Voight model was used to obtain the thickness of the film. Overtone combination of 5, 9, and 11 or 3, 7, and 13 were used for Voight model. For viscoelastic layers, the fluid density and viscosity was assumed to be 1000 kg/m^3 and 0.001 kg/m/s, respectively, while the density of the adsorbed layer for protein with trapped water was assumed to be 1200 kg/m^3 (Liu, et al., 2009).

4.2.2 Statistical Analysis

One-way ANOVA was performed on the results of layer thickness and mass using SPSS 22 (IBM) at a significance level of 5%.

4.3 Results and Discussion

4.2.1 Adsorption of protein and Influence of protein concentration

Whey protein isolate is an amphiphilic material which is capable of adsorbing to the surface of oil droplets and stabilizing the oil droplets in the aqueous phase. In order to imitate oil in water emulsions, a modification of the sensor surface was performed. Gold sensors coated with 1-hexadecanethiol to form a hydrophobic layer were used in this study (Teo, et al., 2016). Coating of the sensor increased the contact angle of the sensor to 89°. In this part, WPI solutions with different concentrations, namely 0.33%, 0.50%, and 1.00% were prepared in a sodium phosphate buffer (pH 6.55). It has to be noted that whereas emulsification involves high shear rates, a high interfacial area to volume ratio, and a short processing time, the adsorption experiment performed using QCM-D is mainly driven by the diffusion of the biopolymers, has a much smaller interfacial area to volume ratio, and is performed at much longer contact times, which makes a direct comparison between both situations troublesome.

Figure 4.2 depicts the frequency and dissipation shift as a function of time for the 3rd, 5th, and 7th overtone upon introduction of WPI to the surface of the hydrophobised gold sensor at different WPI concentrations (0.33%, 0.50%, and 1.00%). The measurement was started by acquiring a stable baseline for approximately 20 minutes. Afterwards, the WPI solution was introduced until a steady state condition (i.e. no further adsorption) was obtained. The experiment was finalized by rinsing the sensor with buffer to remove the loosely attached protein. It can be seen that for all samples, there was a sudden increase in the dissipation accompanied by a decrease in the frequency. The formation of a multilayer on the surface of the sensor could be the cause of the high energy dissipation (Kim, Weber, Shin, Huang, & Liu, 2007). This multilayer is soft and viscoelastic, which dissipated more energy leading to an increase in the magnitude of the dissipation. On the other hand, the decrease on the frequency indicated adsorption on the surface of the sensor. Upon rinsing, the frequency was recorded to increase, while the dissipation decreased. This implies that there was some mass removal from the surface of the sensor and a slight change in the viscoelasticity of the layer, whereby the layer seemed to be less viscoelastic now that the loosely attached protein was removed. Globular proteins like WPI are able to form multiple layers on the surface of oil droplets when the concentration of the protein is sufficiently high (Tcholakova, Denkov,

Sidzhakova, Ivanov, & Campbell, 2003). In addition, these authors stated that this multilayer can be removed by rinsing the oil droplet. This phenomenon can be observed clearly in Figure 4.2.

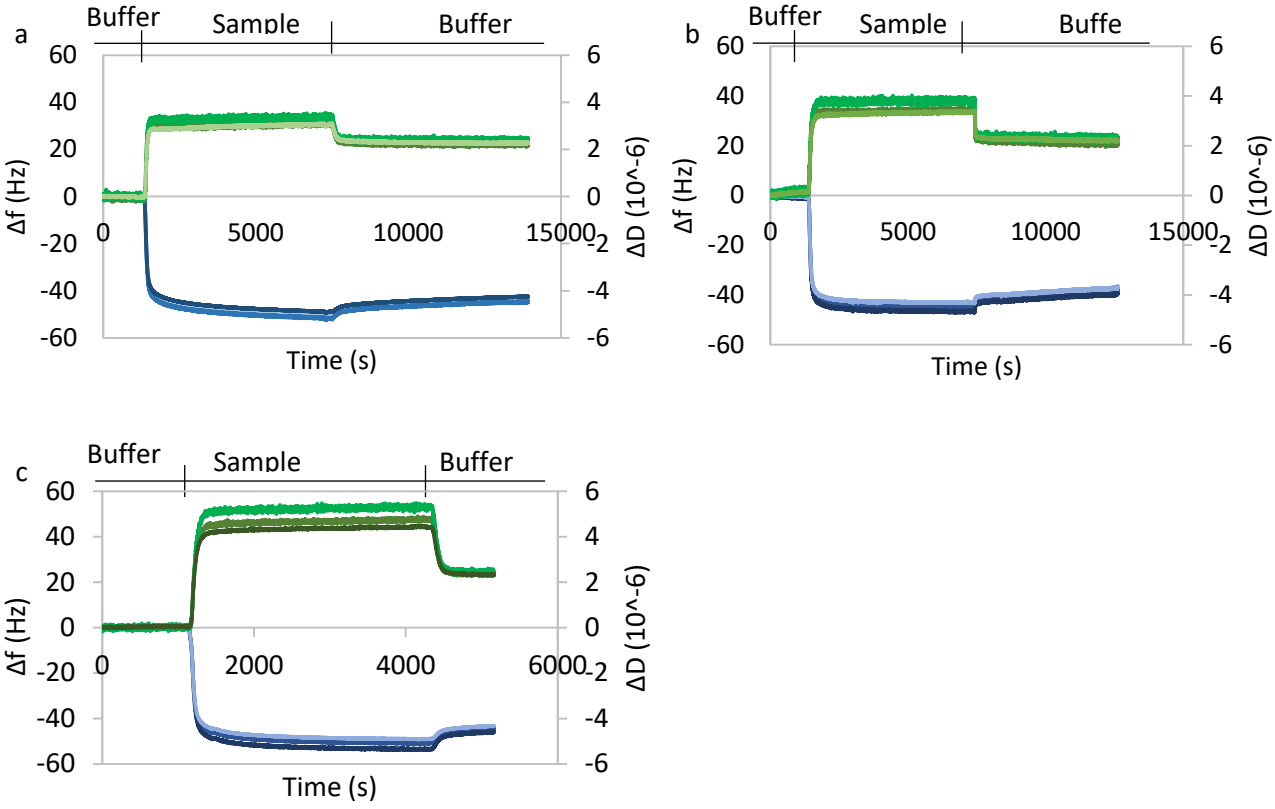


Figure 4.2. Frequency and dissipation shift (of overtone 3, 5, and 7) of WPI adsorption over time as a function of WPI concentration (a. 0.33%, b. 0.50%, c. 1.00%) at pH 6.55 (blue: frequency, green: dissipation).

With regards to the data processing obtained from QCM-D, the magnitude of the frequency is directly proportional with the thickness of the formed layer in the case of a rigid layer. Therefore, the thickness and surface load can be directly obtained using Equation 4.1 and 4.2. Hereby, Δm is the areal mass density (g/cm^2), i.e. the amount of mass adsorbed per cm^2 of the sensor area; $\frac{\Delta F}{n}$ is the change in the resonance frequency; C is the fundamental resonance frequency (which is 18 ng/(cm^2.Hz) for a gold sensor); ρ_f is the density of the adsorbed layer (kg/m^3); and lastly h_f is the thickness of the adsorbed layer (m) (Reviakine, Johannsmann, & Richter, 2011). A rigid layer is characterized by having an equal normalized frequency at all

overtones and by a dissipation shift that is smaller than $1 \cdot 10^{-6}$ (Kim, et al., 2007; Österberg, et al., 2007).

$$\Delta m = C \frac{\Delta F}{n} \quad \text{(Equation 4.1)}$$

$$\Delta m = \rho_f \, x \, h_f \quad \text{(Equation 4.2)}$$

$$\Delta f = -\frac{1}{2\pi\rho_0 h_0} \left\{ \frac{\eta_3}{\delta_3} + h_1 \rho_1 \omega - 2h_1 \left(\frac{\eta_3}{\delta_3}\right)^2 \frac{\eta_1 \omega^2}{\mu_1^2 + \omega^2 \eta_1^2} \right\} \quad \text{(Equation 4.3)}$$

$$\Delta D = \frac{1}{\pi f \rho_0 h_0} \left\{ \frac{\eta_3}{\delta_3} + 2h_1 \left(\frac{\eta_3}{\delta_3}\right)^2 \frac{\eta_1 \omega}{\mu_1^2 + \omega^2 \eta_1^2} \right\} \quad \text{(Equation 4.4)}$$

Whereas the Sauerbrey equation can be used to calculate the thickness of a rigid layer, this equation is not valid for a viscoelastic layer since it will underestimate the surface load (adsorbed mass) and thickness of a viscoelastic layer (Dixon, 2008). Instead, the thickness of a layer with viscoelastic properties must be obtained using the Voight model (equation 4.3 and 4.4), where ρ_0 and h_0 are the density and thickness of the crystal, η_3 is the viscosity of the bulk liquid, δ_3 is the viscous penetration depth of the shear wave in the bulk liquid, ρ_3 is the density of liquid, ω is the angular frequency of the oscillation, whereas $\rho_1, \eta_1, \mu_1, \delta_1$ represent the density, viscosity, shear elasticity, and thickness of the adsorbed layer, respectively. In the Voight model, the dissipation value is taken into consideration in the calculation and gives information on the viscoelastic properties of the adsorbed layer (Eronen, Junka, Laine, & Österberg, 2011). The surface load or the adsorbed mass of a viscoelastic layer will also include a contribution from the liquid in the interior of the compound and in interstitial spaces between the adsorbed compound in which the amount is influenced by the surface coverage, size, shape, orientation, and lateral distribution of the adsorbate (Reviakine, et al., 2011).

Globular proteins such as whey protein tend to form a more viscous and rigid layer than disordered proteins (caseinate) and low molecular-weight surfactants (Lefèvre, et al., 2003). Based on Fig. 4.2, it can be observed that the layer formed by WPI was rather viscoelastic ($\Delta D > 1 \times 10^{-6}$), irrespective of the concentration. According to its dissipation value, the layer was not very viscous, but also not very highly viscoelastic. Therefore, the thickness of the layer was calculated using the Voight model (Table 4.1). Nevertheless, for a comparison, the thickness of the layer obtained using the Sauerbrey equation is also presented in Table 4.1. It can be seen that the value obtained by Sauerbrey is smaller than that obtained with the Voight

model. As for the thickness of the layer before rinsing, there was a high overtone dependency and the difference in the results obtained via the Sauerbrey equation and the Voight model was very significant (more than 15%). Hence, it can be observed that the more viscoelastic a layer is, or the bigger the dissipation value is, the bigger the difference between the values obtained via the two methods will be.

Analysis of Variance (ANOVA) revealed that the thickness of the layer was comparable at all WPI concentrations ($p>0.05$). Increasing the concentration of the WPI did not increase the thickness of the layer formed on the hydrophobic surface. It has been reported that a monolayer with a thickness of 4.95 nm was obtained from WPI adsorption on a hydrophobicaly modified gold sensor at pH 6 (Teo, et al., 2016). In different literature reports, it was mentioned that the length of β-lactoglobulin is approximately 6.93 nm in a dimer state (Kinsella, et al., 1989). β-lactoglobulin exists as a dimer at around neutral pH (Nielsen, Singh, & Latham, 1996). Since the experiment was performed at pH 6.55, the WPI solution prepared contained mostly β-lactoglobulin in a dimeric state. Hence, compared to the diameter of a dimer of β-lactoglobulin found in the literature, it seemed that the layer formed in this experiment was a layer constructed of dimeric β-lactoglobulin. However, it should be taken into account that the sample used in this experiment was WPI and not pure β-lactoglobulin, meaning that there were other compounds such as α-lactalbumin which could be present in the adsorbed layer.

Figure 4.3 shows that Sauerbrey equation underestimated the surface load of the adsorbed layer. The result implies that the adsorbed mass was not influenced by the concentration of WPI ($p>0.05$). With the same surface area available for protein adsorption, it seems that a higher WPI concentration resulted in more unadsorbed protein. Swalinski. et al. (2003) conducted a research by producing WPI stabilized emulsions with the same droplet size ($d_{3.2}$) while varying the concentration of WPI. The result is in agreement with our finding in which the amount of unadsorbed protein increased as the concentration of WPI increased. Protein adsorption, which is expressed as the amount of protein per m^2 of oil droplet surface, is divided into two regions (McClements, 2004; Tcholakova, Denkov, Ivanov, & Campbell, 2002; Tcholakova, et al., 2003). At protein concentrations below a critical concentration, the protein forms a monolayer and the protein load (surface load) is not influenced by the concentration of protein in the emulsion. On the other hand, above the critical concentration, a multilayer is

built on top of the monolayer and protein adsorption is influenced by the concentration of the protein. In this case, the critical concentration is different for each emulsion depending on the hydrodynamic conditions of the emulsion (Tcholakova, et al., 2003). A WPI monolayer on the surface of oil droplets is described to have a surface load of 1.6-2.0 mg protein/m^2 (Tcholakova, et al., 2002; Tcholakova, et al., 2003). The same authors stated that the formation of a multilayer on top of a monolayer leads to a higher surface load and that this multilayer is reversibly attached; thus, it can be easily removed by rinsing. Based on the Voight model, the surface load of WPI at all concentrations was approximately 9.0 mg/m^2. Hence, considering the thickness of the layer and the viscoelasticity of the layer, the high surface load obtained by the QCM-D in this study can be concluded to be due to the formation of a multilayer. A high protein surface load was also reported by Sünder et al. (2001): at 1-2% WPI concentration, the surface load of WPI stabilized emulsions was found to be approximately 6.3 mg/m^2.

While it has been mentioned that at high protein concentration, the protein load is influenced by the protein concentration, the results show that after rinsing the final surface load was comparable at all WPI concentrations studied. Nevertheless, It should be kept in mind that the adsorbed mass obtained includes the mass of the solvent or liquid trapped inside the film. Thus, the surface load obtained from the QCM-D results from both the WPI and the trapped liquid. In order to obtain the surface load of protein only (adsorbate), the contribution of the trapped liquid should be eliminated. Therefore, to do a direct comparison with the surface load obtained from protein determination on the surface of oil droplets, an additional measurement to obtain the protein load on the surface of the sensor, via for instance ellipsometry, is needed. Nonetheless, the results from QCM-D still can nicely illustrate the phenomenon of WPI adsorption on the surface of oil droplets as well as giving valuable information on the properties of the layer.

Table 4.1. Thickness (nm) of WPI layers formed at the surface of modified gold sensors at pH 6.55 calculated using the Sauerbrey equation (7th overtone) and the Voight model.

WPI concentration	Sauerbrey		Voight model	
	Before rinsing	After rinsing	Before rinsing	After rinsing
0.33%	7.87±0.41^a	6.80±0.34^a	9.90±0.25^a	7.98±0.14^a
0.50%	7.56±0.36^a	6.72±0.26^a	9.82±0.48^a	7.74±0.36^a
1.00%	7.79±0.76^a	6.43±0.62^a	11.29±1.09^a	7.31±0.63^a

^{a,b} Means in the same column followed by different lowercase letters are significantly different ($p<0.05$).

Figure 4.4 shows the frequency versus dissipation profile of WPI adsorption at different WPI concentrations to illustrate the adsorption process of WPI onto the surface of the sensor, independent of time, and to compare the properties of the adsorbed layer (Kontturi, et al., 2008; Park, et al., 2017). The graph reveals two different slopes, in which the first slope indicates the adsorption rate of the sample while the second one gives an idea of the conformation of the protein layer (Kim, et al., 2007). The result presented in Fig. 4.4 shows that the adsorption rate of WPI was not influenced by the concentration of WPI as they all had comparable slopes (1st slope). At this stage, the dissipation value increased linearly with the frequency. After a rapid adsorption of WPI (1st slope), the adsorption proceeded at a slower rate (2nd slope). At this stage, the slopes at a WPI concentration of 0.50 and 0.33% were almost zero. The graph shows that 1.00% WPI reached a higher dissipation value than the rest, while 0.50% WPI reached a slightly higher dissipation value than 0.33% WPI. The high dissipation magnitude obtained at this stage implies that the layer dissipated more energy per frequency unit which indicates that the layer is viscoelastic and soft (Kontturi, et al., 2008). The viscoelasticity of the layer might be caused by multilayer formation on the surface of the sensor, which was more preferable at a high concentration of WPI. Nevertheless, this layer was removed during rinsing (Fig. 4.3). As a consequence, the Δf/Δd of 1.00% WPI was comparable with that of 0.33% and 0.50% WPI at the end of the experiment.

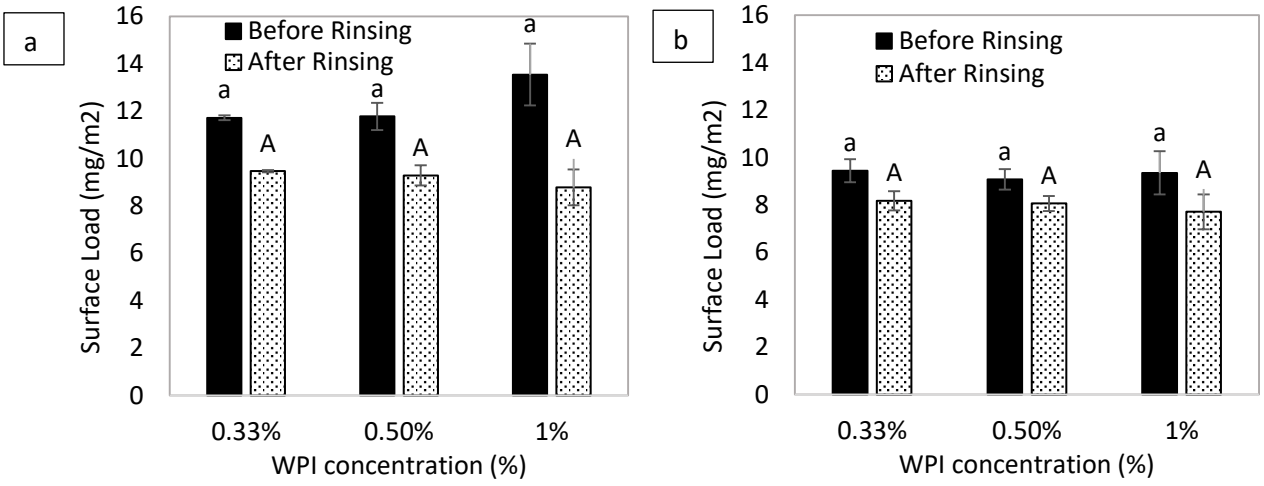


Figure 4.3. Adsorbed mass (mg/m^2) of the WPI layer obtained with the Sauerbrey (a) and the Voight model (b), both before and after rinsing the sensor with WPI-free buffer. Samples with the same letter show no statistical difference between them.

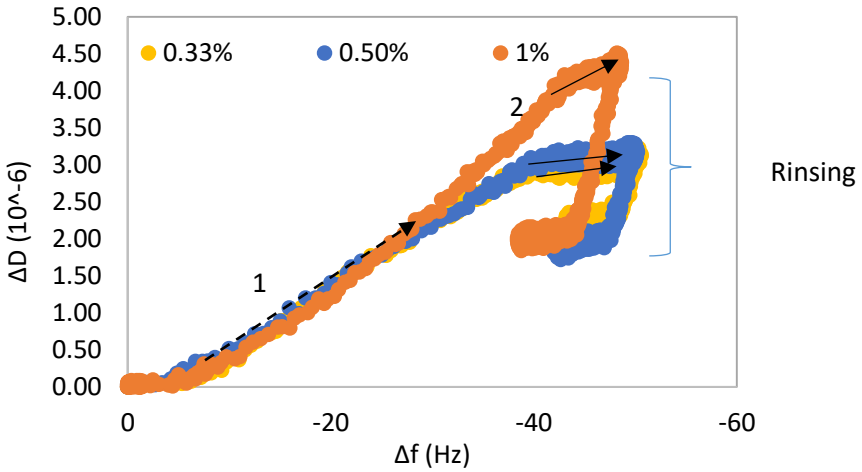


Figure 4.4. Frequency versus dissipation profile of WPI adsorption at pH 6.55 at different WPI concentrations. The graph was created using data from the 7th overtone.

4.2.2 Influence of pH on WPI adsorption

Since pH 5.0 is known to be close to the IEP of WPI, protein aggregation is expected. At pH 5.0, protein aggregation is favoured since WPI has a very low net charge density (close to zero). Due to this phenomenon, WPI aggregates were observed in the prepared WPI solution. Thus, constant stirring was required during the experiment to avoid sedimentation of the WPI. The influence of pH on the adsorption of WPI can be seen in Fig. 4.5. The pH influenced the arrangement of WPI on the surface of the sensor. β-Lactoglobulin was reported to exhibit a flexible and open structure at pH values above 6 and a more rigid and compressed structure at low pH, such as at pH 2 (Benjamin, Lassé, Silcock, & Everett, 2012). Whereas an expanded and less rigid film is obtained when there is a high net charge, which inhibits protein-protein interaction, a rigid and condensed layer is obtained in an environment where the net charge is low (i.e. around the IEP of the protein) due to maximum protein-protein interaction and a high adsorption rate (Kinsella, et al., 1989). However, the latter case is only applicable if the protein remains soluble. Fig. 4.5b depicts that the adsorption of WPI at pH 6.55 was faster than at pH 5.0. This could be due to the fact that at pH 5.0 the protein was aggregated and thus diffused slower to the surface of the sensor than the non-aggregated protein.

It can be seen that the layer formed at pH 6.55 dissipated more energy per frequency unit than that at pH 5.0 (Fig. 4.5b) indicating a soft and viscoelastic layer. High Δf/ΔD values can indicate the presence of a high amount of liquid trapped in the adsorbed layer (Kontturi, et al., 2008). Upon adsorption of WPI, the proteins will be strongly adsorbed at the hydrophobic

surface forming a monolayer. At excess amount of protein, a multilayer is formed because adsorption of protein on the surface of the monolayer is obtained and the configuration of this layer is influenced by pH. As it was mentioned previously, at pH 6.55 the attraction force between proteins is rather low. Therefore, the protein-protein interaction was weak and the structure of the layer was rather loose; as such, liquid can be trapped in the layer. Fig. 4.5.b also indicates that the layer at pH 5.0 was more rigid. At a pH around its IEP, WPI has a weak electrostatic repulsion and exposes its hydrophobic groups which induce protein-protein interaction (Ju, et al., 1998). A strong protein-protein interaction results in a compact structure, thus a rigid layer with a low amount of trapped liquid is obtained.

Table 4.2 shows the thickness of the adsorbed layer, the adsorbed mass and the dissipation of the adsorbed layer obtained at pH 6.55 and 5.0. While the layer at pH 5.0 was more rigid than that at pH 6.55, the thickness of the layer was calculated using the Voight model since the dissipation value was a little above 1 x 10^{-6}. According to the Voight model, the thickness of the adsorbed layer and the adsorbed mass at pH 6.55 were higher than that at pH 5.0. Based on this result, it seems that at pH 6.55 the configuration of the layer was more extended than that at pH 5.0 in which it was more compact and rigid. As it has been mentioned, for viscoelastic layers, the trapped liquid in the adsorbed layer contributes to the surface coverage (area mass density). Therefore, considering the viscoelasticity of the layer, it might be that the amount of WPI (adsorbate) adsorbed at pH 5.0 was in fact higher than that at pH 6.5 since at pH 5.0 the layer incorporated less liquid (Fig. 4.6). A layer with a high amount of trapped liquid tends to have a lower surface load of the adsorbate (Reviakine, et al., 2011). Shimizu et al. (1981) reported that there was 7.6 mg/m^2 of protein on the interface of oil droplets at pH 5.0, determined via Lowry analysis. This result was similar to the surface load reported in this study (Table 4.2). This might indicate that for rigid layers, the obtained surface load from QCM-D is comparable to the surface load obtained from chemical analysis due to the low contribution from the trapped liquid. A previous study on the stability of WPI stabilized emulsions revealed that emulsions stabilized by WPI at pH 5.0 (in the presence of 30 mM NaCl) had a higher protein load than that at pH 6.55 (Setiowati, Saeedi, Wijaya, & Van der Meeren, 2017). The emulsion prepared at pH 6.55 was also found to have a better stability. The stability of the emulsions at pH 6.55 can be attributed to the presence of a viscoelastic film which can protect the oil droplets from compression and distortion (Kinsella, et al., 1989). In addition to

the electrostatic stabilization, this viscoelastic layer might also be responsible for the steric stabilization of the WPI stabilized emulsions.

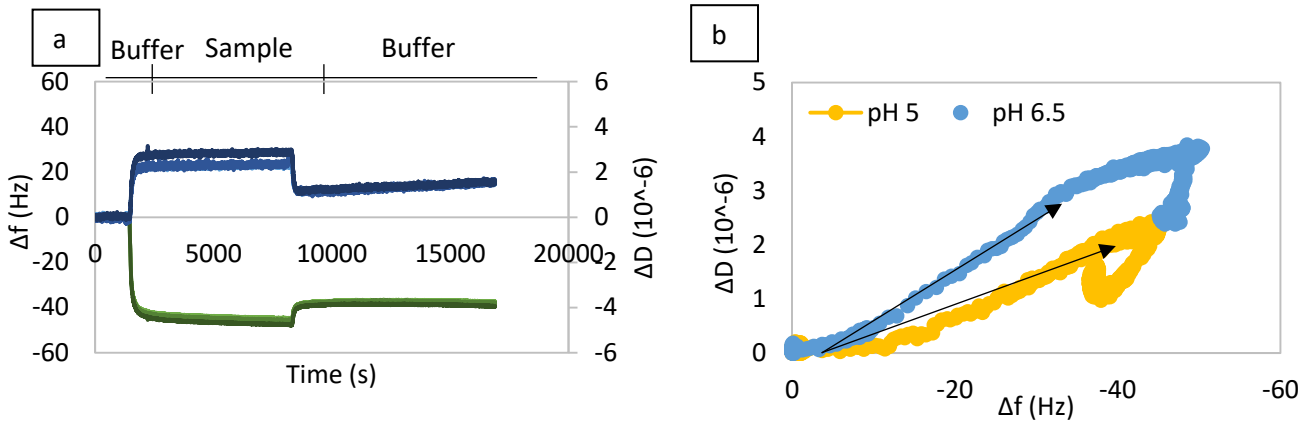


Figure 4.5. Frequency and dissipation shift (overtone 3, 5, and 7) of WPI (0.33%) adsorption over time at pH 5.0 (green: frequency, blue: dissipation) (a) and Δf vs ΔD profile of WPI adsorption at pH 5.0 and pH 6.5 (b) on a hydrophobic gold surface.

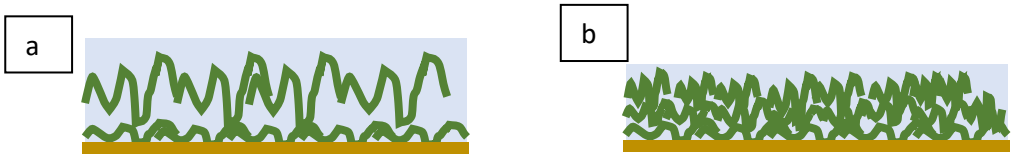


Figure 4.6. Schematic structure of the WPI adsorbed layer at pH 6.55 (a) and pH 5.0 (b) on the surface of a hydrophobic gold sensor (green: protein, blue: trapped water).

Table 4.2. Thickness, adsorbed mass, and dissipation of the adsorbed layer (after rinsing) obtained from 0.50% WPI prepared at pH 6.55 and 5.0 obtained with the Voight model.

pH	ΔD (1 x 10^{-6})	Thickness (nm)	Adsorbed mass (mg/m^2)
6.55	2.15±0.25	7.74±0.36	9.29±0.43
5.0	1.69±0.08	6.39±0.44	7.65±053

Additional experiments were performed to have a better observation on the response of an adsorbed layer when it is exposed to a lower pH (acidic environment). In this experiment, the WPI solution was introduced to the sensor at pH 6.55 which was then followed by rinsing the sensor with a protein-free buffer at the same pH. Subsequent rinsing was then performed with

another protein-free buffer at lower pH. Through this method, the formation of protein aggregates in the solution at low pH can be avoided. Furthermore, this technique had an advantage in which it was possible to observe the change in the configuration of the adsorbed layer when it was exposed to a lower pH. Fig. 4.7 shows the frequency and dissipation profile of WPI adsorption at pH 6.55 followed by rinsing at pH 5.0 (a) and 4.5 (b).

At pH 6.55, it can be seen that the WPI adsorbed layer tended to be rather viscoelastic. Changing the pH of the environment to a lower pH, apparently strongly influenced the properties of the adsorbed layer. There was a noticeable decrease in the dissipation and an increase in the frequency as the layer was rinsed with buffer at pH 4.5 and 5.0. Besides implying that there was mass removal from the surface of the sensor, it could also imply that there was a change in the configuration of the adsorbed layer at the surface of the sensor. The layer was transformed to a more rigid layer when the pH was reduced. The fact that the frequency and dissipation shifts became much less dependent on the overtone (Fig. 4.7) shows that the adsorbed layer was rigid. This transformation was suspected to be accompanied by the release of trapped liquid from the adsorbed layer. Table 4.3 shows that by decreasing the pH, the thickness of the adsorbed layer decreased by about 3 nm, while the surface load decreased by approximately 3.0 mg/m^2. When the pH is reduced approaching its IEP, the interaction between proteins in the adsorbed layer is enhanced. The proteins in the adsorbed layer became strongly attracted to each other and the liquid trapped within the layer is then released resulting in a compact structure and a less extended layer. Therefore, the reduction of the surface load (Table 4.3) observed after rinsing at a lower pH could not only be attributed to a possible loss of WPI from the surface of the sensor, but also to liquid removal from the layer due to a change in the configuration of the adsorbed layer. Table 4.3 shows that adsorption of WPI at a pH slightly above and below the IEP of WPI produced a layer with comparable properties. Fig. 4.7d illustrates the change in the adsorbed layer configuration. This implies that when an emulsion is exposed to a different pH, the response of the layer will be highly influenced by the pH. Decreasing the pH from a relatively neutral pH to a pH close to the IEP of WPI changed the adsorbed layer from a viscoelastic to a rigid layer. This layer regained its viscoelasticity when the pH was brought back to a pH far from its IEP (Fig. 4.7c). This phenomenon was accompanied by an increase in the frequency indicating that there was additional water trapped between the adsorbed layers.

Table 4.3. Thickness and adsorbed mass of the adsorbed layer obtained from 0.33% WPI at pH 6.55 followed by rinsing at pH 6.55 and 4.5/5.0 obtained with the Voight model.

pH	Thickness (nm)		Mass (mg/m^2)	
	1st rinsing	2nd rinsing	1st rinsing	2nd rinsing
6.55-4.5	7.92±0.14	5.27±0.34	9.50±0.17	6.72±0.38
6.55-5.0	7.94±0.30	5.47±0.15	9.53±0.36	6.57±0.18

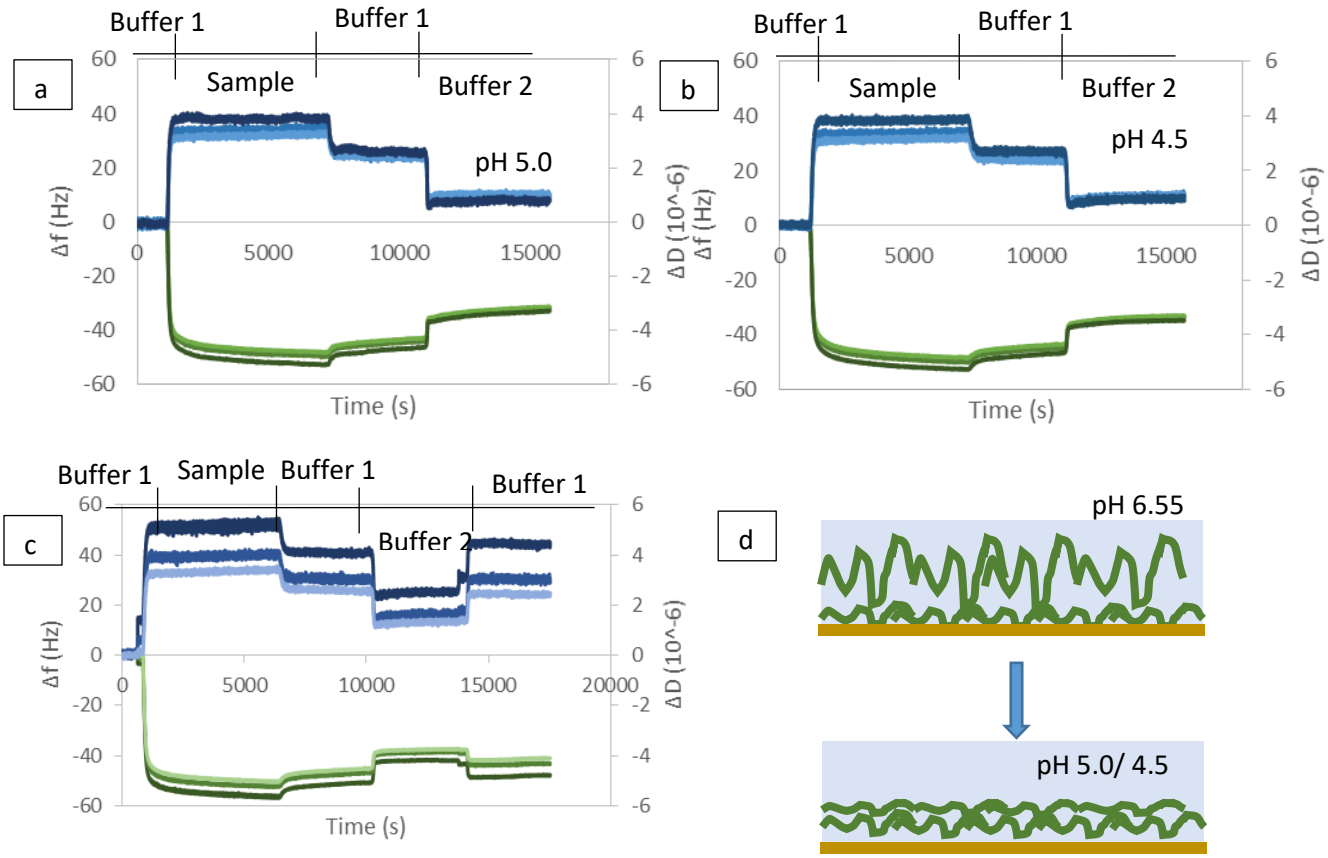


Figure 4.7. Frequency and dissipation shift (overtone 3, 5, and 7) of WPI (0.33%) adsorption over time at pH 6.55 followed by rinsing at a) pH 5.0, b) pH 4.5 (blue: dissipation; green: frequency), c) pH 5.0 continued by final rinsing at pH 6.5 and d) the schematic structure of the adsorbed layer.

4.2.3 Interaction of WPI and LMP

This part deals with the interaction between WPI and LMP at pH 6.55 and 5.5. For this part, a (0.33%) WPI and (0.17%) LMP solution was introduced to the sensor sequentially whereby rinsing was performed in between. The WPI and LMP solutions were prepared separately at

the same pH. The sequence of the experiment started by pumping the buffer solution to obtain a stable baseline, followed by introducing the WPI solutions. Subsequently, the WPI layer was rinsed and LMP was introduced to the WPI layer. Finally, the layer was rinsed once again with the same buffer.

The interaction of WPI and LMP at pH 6.55 and pH 5.5 are presented in Fig. 4.8. Similar to the previous results, as WPI was pumped to the sensor, there was a sudden increase in the dissipation and a decrease in the frequency. The first rinsing removed all the loosely attached WPI from the surface of the sensor leaving only the strongly attached WPI. Afterwards, when the LMP was introduced, the phenomenon was highly influenced by pH. At a relatively neutral pH, when the LMP was introduced to the WPI layer there was a prominent increase in the dissipation of the sensors without significant reduction of the frequency. This phenomenon was suggested to be due to a change of the properties of the bulk liquid (Kontturi, et al., 2008) since the LMP solution had a higher viscosity than the buffer solution. Changing the bulk liquid to WPI- and LMP-free buffer during rinsing decreased the dissipation to its original magnitude before rinsing. As it can be observed, no noticeable change was observed in the frequency when the LMP was introduced. It was found that the thickness and surface load of the layer was comparable before and after LMP addition, while the thickness slightly decreased, which might be due to rinsing. Based on this, it can be concluded that there was no interaction of LMP with the adsorbed layer of WPI at pH 6.55. This indicates that at this pH the interaction between WPI and LMP was not favourable.

A different phenomenon was observed when the experiment was performed at pH 5.5. Fig 4.8.b nicely illustrates the interaction between WPI and LMP at this pH. When the LMP was introduced, there was an increase in the dissipation accompanied by a decrease in the frequency. Besides due to the change in the bulk properties, it also showed that there was some mass adsorption. The frequency did not increase after rinsing suggesting that the adsorbed mass was not removed by rinsing. The dissipation decreased after rinsing; nonetheless, the dissipation was still higher than before the LMP was introduced.

The thickness of the WPI layer before and after the introduction of LMP is presented in Table 4.4. It can be seen that at pH 5.5 the thickness and surface load of the WPI layer was slightly lower than that formed at pH 6.55. Moreover, at pH 5.5, the layer was more rigid and

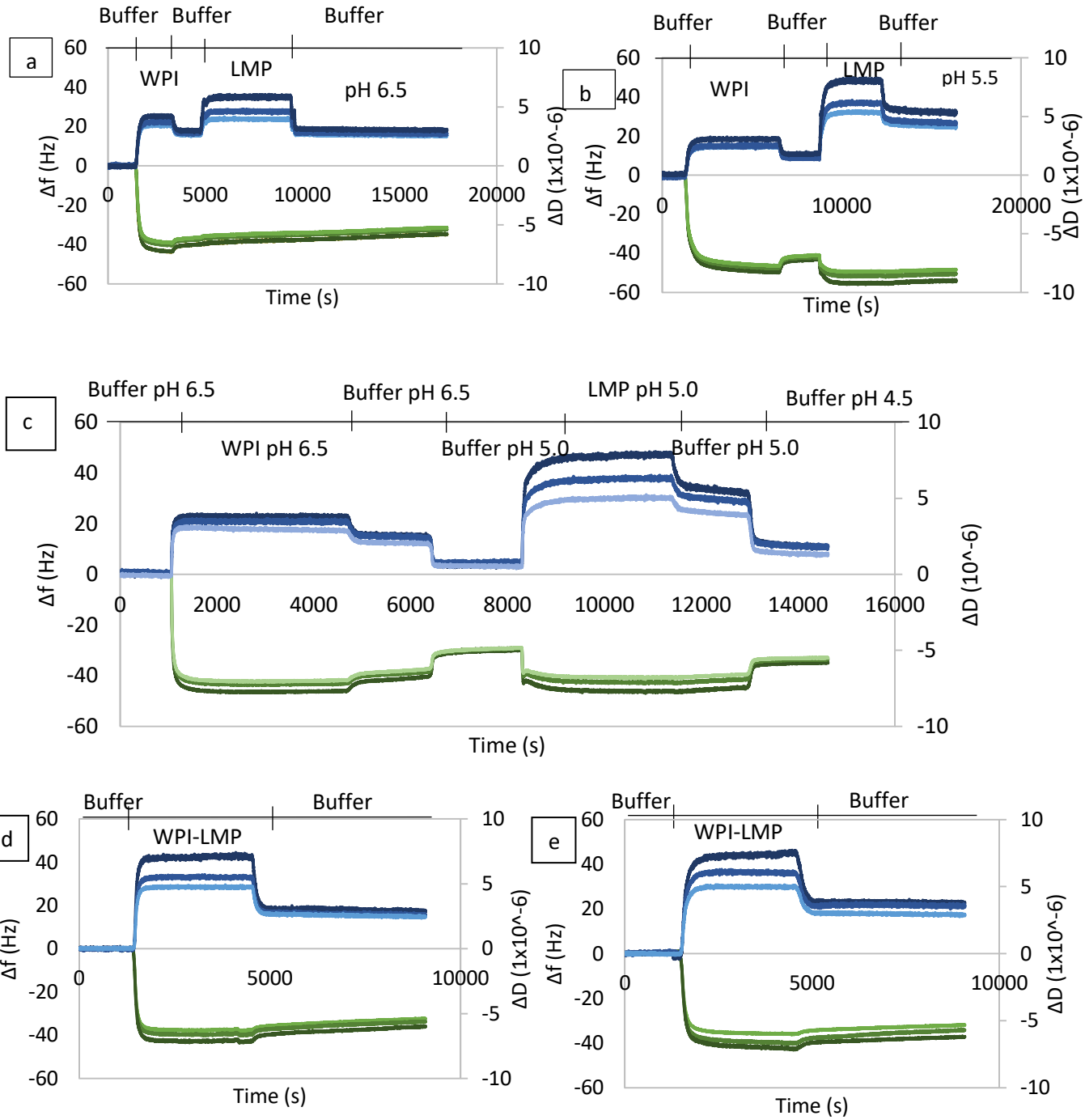
dissipated less energy (Fig. 4.8e). This means that the layer contained less liquid and thus a higher protein load was possible due to stronger protein-protein attraction at pH 5.5 than at pH 6.55. Upon addition of LMP, the thickness of the layer grew from 7.53 nm to 12.75 nm. This was also accompanied by an increase of the surface load by approximately 5.0 mg/m^2.

The final adsorbed layer obtained at pH 5.5 was more viscoelastic and thicker than that at pH 6.55 (Table 4.2) due to the presence of bound LMP. The final layer was shown to have a strong overtone dependence. It can be observed that the layer formed by LMP was much more viscoelastic and dissipated more energy. The amount of liquid trapped in the adsorbed layer can be roughly estimated from Figure 4.8e (Kontturi, et al., 2008). Due to the hydrophilicity of LMP, it might carry a high amount of liquid when it interacted with WPI. Thus, the amount of LMP that interacted with WPI was most likely less than 5.0 mg/m^2. The schematic structure of the layer can be found in Figure 4.8g.

These results, however, confirm the formation of WPI-LMP electrostatic complexes at pH 5.5. This was possible due to the different charge between WPI and LMP which would not occur at a pH where both biopolymers had the same charge (i.e. around neutral pH) (Harnsilawat, et al., 2006a). It was then predicted that oil droplets in an emulsion stabilized by WPI-LMP at pH 5.5 will exhibit a better stability than those stabilized by WPI-LMP at pH 6.55. At pH 5.5, the oil droplets will have a thicker and more viscoelastic layer which protects the oil droplets from destabilization. On the other hand, at pH 6.55, free LMP will be present in the aqueous phase and depending on its concentration can induce droplet flocculation through depletion flocculation.

In addition, since QCM-D is sensitive enough to the method applied, the response of these WPI-LMP complex layers to a pH change can also be studied by changing the pH of the adsorbed layer. For instance, Fig. 4.8c shows the formation of WPI-LMP complexes on the surface of the sensor by sequential adsorption at pH 5.0. It can be observed that by decreasing the pH to pH 4.5, there was a decrease in the dissipation and frequency. This was unlikely due to the removal of the adsorbed layer (desorption), this was suggested to be due to the re-arrangement of the WPI-LMP complex structure. It has been reported that the attraction between WPI and pectin was stronger at a pH below the IEP of WPI as the number of the cationic groups on the surface of the protein increased (Salminen, et al., 2014). Due to this,

some water might be released from the adsorbed layer which explained the reduction in the frequency and dissipation of the adsorbed layer.



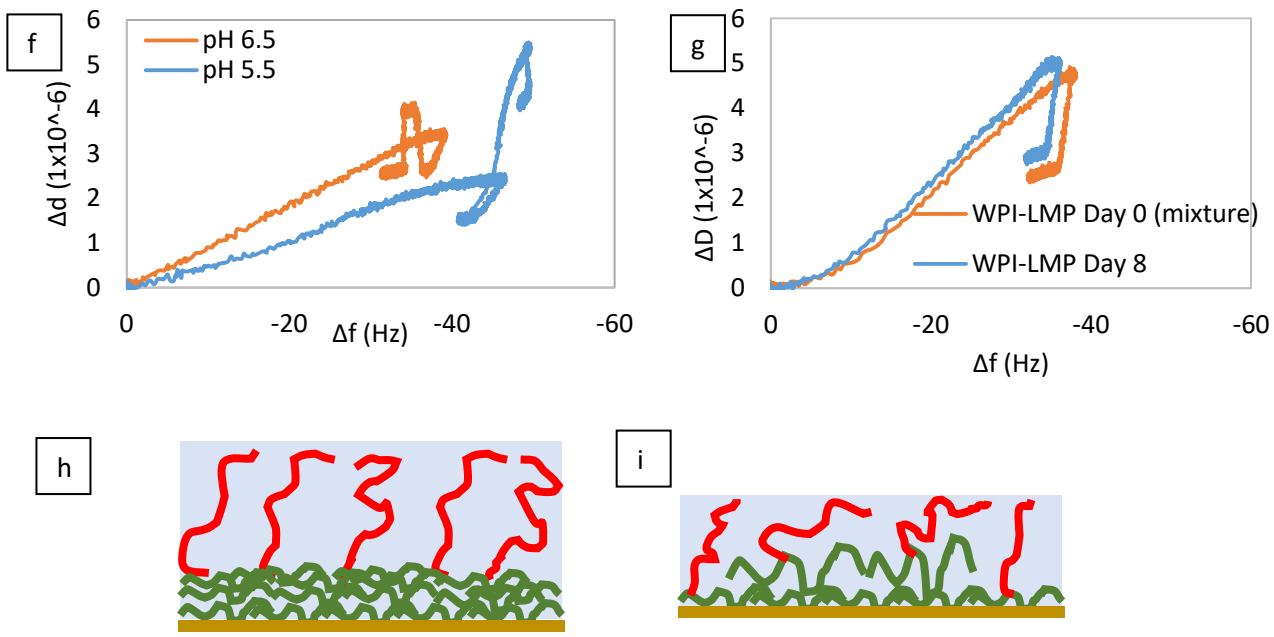


Figure 4.8. Frequency and dissipation shift of sequential adsorption of WPI (0.33%) and LMP (0.17%) over time at a) pH 6.55, b) pH 5.5, c) Frequency and dissipation shift of sequential adsorption of WPI and LMP over time at different pH values, d) WPI-LMP Day 0 (mixture) pH 6.55, e) WPI-LMP conjugate Day 8 at pH 6.55 over time (blue: dissipation; green: frequency). f) Δf/ΔD profile of WPI-LMP sequential adsorption at pH 6.55 and 5.5. g) Δf/ΔD profile of WPI LMP Day 0 and Day 8 adsorption at pH 6.55 created using data from the 7th overtone. The schematic structure of the adsorbed layer obtained from WPI-LMP sequential adsorption (h) and from WPI-LMP conjugates (i) is also shown.

Table 4.4. Thickness (nm) and surface load (mg/m^2) of the adsorbed layer obtained with the Voight model. The dissipation value was obtained from the 7th overtone.

Sample	Thickness (nm)		Mass (mg/m^2)		ΔD (1 x 10^{-6})	
	WPI	WPI+LMP	WPI	WPI+LMP	WPI	WPI+LMP
Sequential WPI-LMP pH 6.55	7.90±0.32	7.68±0.56	9.48±0.54	8.45±0.62	2.50±0.03	2.35±0.12
Sequential WPI-LMP pH 5.5	7.53±0.78	12.8±0.9	9.04±0.93	14.0±1.0	1.50±0.06	4.03±0.13
WPI-LMP Day 0 (Mixture) pH 6.55	8.00±0.34	-	9.60±0.41	-	2.49±0.06	-
WPI-LMP Day 8 pH 6.55	8.38±0.20	-	10.1±0.23	-	2.89±0.06	-

4.2.4 Mixture of WPI-LMP vs conjugated WPI-LMP

For a comparison, WPI-LMP mixture adsorption was performed. Instead of layer by layer adsorption, this experiment was performed by preparing a solution containing 0.5% of a mixture of WPI and LMP (WPI:LMP ratio 2:1). The solution was then pumped into the sensor chamber and followed by rinsing. Through this method, it was found that the adsorbed layer had a thickness of 8 nm and a surface load of 9.6 mg/m^2 (Table 4.4). The thickness obtained was comparable to the thickness of a WPI layer obtained by sequential adsorption at pH 6.55, while the load was slightly higher. It was possible that due to its molecular weight and hydrophilicity, the LMP diffused slower to the surface of the sensor and adsorption of LMP was not favourable. Furthermore, the pH was not favourable for the LMP to interact with the WPI.

Adsorption of WPI-LMP conjugates which has been dry heat treated for 8 days resulted in a thicker layer than that formed from a WPI-LMP mixture, albeit the difference was small. The higher surface load of the WPI-LMP conjugate layer might come from the trapped liquid carried by the covalently linked LMP. Dry heat treatment of WPI and LMP has been proved to produce high molecular weight compounds as a result of covalent interaction between WPI and LMP through Maillard type reaction (Setiowati, et al., 2016). Therefore, the WPI-LMP conjugate solution would contain a mixture of high molecular weight compounds (WPI-LMP conjugates), free WPI, and free LMP. The adsorption test using QCM-D was able to give information on the properties of the layer such as the thickness, viscoelasticity, as well as its surface load. Nevertheless, it is difficult to determine and identify the compound adsorbed at the surface of the sensor. Based on the WPI-LMP interaction experiment at pH 6.55, it was less likely that the free LMP was present in the layer. Thus, the higher viscoelasticity of the layer could be due to the covalently bound LMP. The presence of LMP could be the reason of the higher dissipation value of the WPI-LMP conjugates layer as well as the higher surface load observed since LMP, as it was discussed before, tends to create a rather soft layer by trapping a lot of liquid which contributes to the surface load. Therefore, it was suggested that the layer was composed of free WPI and WPI-LMP conjugates (Fig. 4.8h).

Despite giving highly important information, additional analyses are still needed to obtain the adsorbate surface load without the contribution from the trapped liquid. Nevertheless, it can

be said that, overall, this method was very suitable to study the interaction between WPI and LMP, especially with the sequential adsorption method. In this study, QCM-D analysis enabled real-time observation and visualization of what happens on the surface of oil droplets, as well as of the change in the properties of the adsorbed layer when it was exposed to a different environment. Figure 4.8 shows how an adsorbed layer changed/responded as a function of pH and when a different compound was introduced. The response of the layer could be nicely monitored using QCM-D. Hence, it is an interesting method to study interaction between two biopolymers and the possible layer that resulted from the interaction. In emulsion research, this information is very useful in order to understand the mechanism of emulsion stabilization and the properties of the film formed by an emulsifier. Furthermore, using this method it will be possible to design a layer with specific properties and thickness to produce a specific emulsion (Teo, et al., 2016).

4.4 Conclusions

It was revealed that WPI tends to form a somewhat viscoelastic layer around neutral pH (pH 6.5). On the other hand, adsorption at pH conditions closer to the isoelectric point (pH 5.0) gave a relatively rigid layer. This was further confirmed by an adsorption test followed by sequential rinsing (pH 6.5 followed by pH 4.5/5.5). There was rearrangement of the adsorbed WPI when it was rinsed with buffer at a lower pH, in which the layer collapsed and transformed into a more rigid layer. Furthermore, these changes were found to be reversible. As the pH was increased, the layer regained its viscoelasticity. Moreover, adsorption experiments at pH 5.0 gave comparable surface loads to the values obtained via chemical analysis found in literature. Thus, it could be concluded that for a rigid layer, the surface load obtained from QCM-D was comparable to that obtained through chemical analysis. However, this was only applicable when the adsorption and rinsing were performed at the same pH and not by sequential rinsing in which the adsorption and rinsing are performed at different pH. By sequential adsorption, it was found that electrostatic interactions between WPI and LMP produced a thicker and more viscoelastic layer, which was suggested to be responsible for the steric stabilization of emulsions. The results revealed that QCM-D was able to give highly useful information such as the viscoelasticity of the adsorbed layer, thickness, adsorbed mass or surface load, and an approximation of the amount of liquid trapped in the adsorbed layer. Nevertheless, in the case of a viscoelastic layer, additional experiments are required to

determine the amount of adsorbate (WPI or LMP only) as the surface load given by QCM-D included the liquid trapped within the layer. While the results from a sequential adsorption experiment could be well interpreted, it was more challenging to interpret the results when a mixture of biopolymers was used, which was the case for WPI-LMP mixtures and WPI-LMP conjugates. Furthermore, additional experiments which were performed showed that the configuration of the adsorbed layer was sensitive to a pH change. Hence, QCM-D is sensitive enough to study the response of the adsorbed layer to a change of pH which might be useful to study and explain the stability of emulsions stabilized by proteins as a function of pH.

CHAPTER 5

STUDY ON THE INTERACTION OF WHEY PROTEIN AND LOW METHOXYL PECTIN USING PFG-NMR

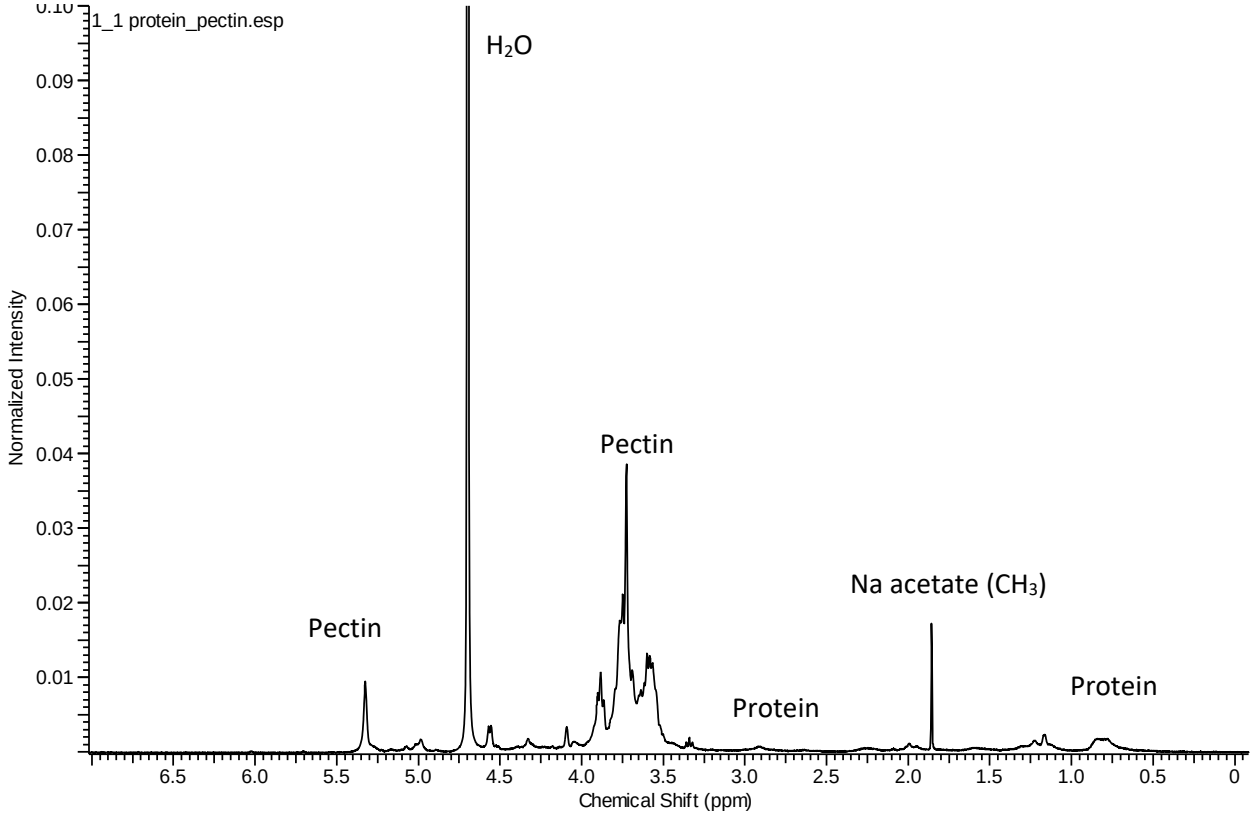


Figure: 1D ^{1}H spectrum of 20 mg/mL (in 5 mM NaAc) 1:1 protein : pectin mixture after 16 days of dry heat incubation

Abstract

In this study, the interaction between WPI and LMP was evaluated using pfg-NMR. The influence of pH, duration of dry heat treatment, and WPI to LMP ratio on WPI and LMP interaction was investigated. While WPI was characterized by its fast diffusion and narrow distribution width, LMP had a smaller diffusion coefficient and broader distribution width. At pH below 7 (e.g.: pH 5.5 and 5.0) WPI-LMP complexes were formed due to electrostatic interaction. Approximately 40-50% of WPI present in the mixture interacted with LMP at pH 5.5 and pH 5.0. As for conjugate measurement, it is important to note that the conjugate measurement should be performed at a pH in which electrostatic interaction between WPI and LMP is limited. Hence, in this study, the measurement of conjugates was performed at pH 7.2. Dry heat treatment of WPI alone did not have a considerable effect on the diffusion behaviour of WPI. However, it is worth to mention that after dry heat treatment of WPI in the absence of LMP, the distribution width of WPI slightly increased. The native and dry heated WPI had a diffusion coefficient of about $7 \cdot 10^{-11}$ m^2/s and were characterized by a narrow distribution width ($1\text{-}2 \cdot 10^{-11}$ m^2/s). In the case of WPI-LMP conjugates, the diffusion coefficient of WPI and LMP were found to be influenced by the duration of the dry heat treatment and the concentration of LMP present in the conjugates. Dry heat treatment of a WPI-LMP mixture caused the diffusion coefficient of WPI to decrease, while the diffusion coefficient of the LMP was only slightly influenced. When mixtures of WPI and LMP were incubated for 1, 2, 8, and 16 days the diffusion coefficient of WPI decreased from approximately $7 \cdot 10^{-11}$ m^2/s in native WPI to $6.5 \cdot 10^{-11}$ m^2/s, $6.42 \cdot 10^{-11}$ m^2/s, $6.27 \cdot 10^{-11}$ m^2/s, and $5.97 \cdot 10^{-11} m^2/s$, respectively. This phenomenon was accompanied by an increase on its distribution width. With regard to the LMP concentration present in the conjugates, the higher the LMP concentration, the higher the amount of WPI bound to LMP, the smaller the diffusion coefficient of WPI/LMP and the broader the distribution width was. The results showed that pfg-NMR is a promising tool to study WPI-LMP interaction.

5.1 Introduction

Nowadays, complexes of proteins and polysaccharides receive a lot of attention due to their high functionality. This type of complexes can be applied in a wide range of products, such as in beverages (Akhtar, et al., 2007; Harnsilawat, Pongsawatmanit, & McClements, 2006b), or gels (Bryant, et al., 1998), or in microencapsulation (Esfanjani, Jafari, & Assadpour, 2017; Teng, Luo, & Wang, 2013). These complexes can be formed either through electrostatic interaction or covalent bonds. Electrostatic interaction involves modification of charge of the biopolymers to facilitate the formation of the complexes and is influenced by different factors such as pH, ionic strength, charge density of the protein and polysaccharides, etc. (Ye, 2008). When the proteins and polysaccharides possess a different charge and exhibit net attraction, protein-polysaccharide complexes is formed (Ye, 2008). There have been a lot of studies on electrostatic interaction between proteins and polysaccharides and its application in emulsions (Benichou, Aserin, & Garti, 2007; Benichou, Aserin, Lutz, & Garti, 2007; Gu, Decker, & McClements, 2005). On the other hand, covalently bound protein-polysaccharide conjugates are normally obtained via Maillard type reactions (Akhtar, et al., 2007; Jimenez-Castano, et al., 2007; Wooster, et al., 2006). This type of conjugates is obtained by exposing a mixture of proteins and polysaccharides to elevated temperatures at certain relative humidity for a certain period of time (Kato, 2002). The presence of heat will induce the amino groups of the proteins to interact with the carboxyl groups of the polysaccharides resulting in covalently linked protein-polysaccharide complexes (Dickinson, 2008). The formation of protein-polysaccharide conjugates can be performed either in dry state (Kato, 2002) or wet state (Diftis, et al., 2005). During preparation, it should be noted that excessive heating can lead to advanced Maillard reaction and cause the conjugates to lose their functionality (Kato, 2002). Therefore, during the preparation of protein-polysaccharide conjugates, the Maillard reaction should be limited to the early stages of Maillard reaction (Amadori arrangement) in order to achieve the desirable result (Oliver, et al., 2006). Previous studies have shown that protein-polysaccharide conjugates had a better functionality than protein/polysaccharides alone (Akhtar, et al., 2007; Jiménez-Castaño, López-Fandiño, et al., 2005; Setiowati, et al., 2016).

In this study whey protein isolate (WPI) and low methoxyl pectin were used to form WPI-LMP conjugates through dry heat treatment (dry state). It has been reported that conjugation of WPI and LMP increased the heat stability of WPI as well as its emulsifying activity (Neirynck, et al., 2004; Salminen, et al., 2014; Setiowati, et al., 2016). Upon emulsification, the hydrophobic groups of whey protein will anchor at the oil part while the hydrophilic parts provided by the polysaccharides will stay in the aqueous phase, thus improving the emulsification of oil droplet (Dickinson, et al., 1992; Diftis, et al., 2006b). Furthermore, the presence of polysaccharides provides an additional layer at the surface of the oil droplets preventing the droplets to come in close vicinity which can lead to phase separation (Zhu, et al., 2010). This will prevent droplet flocculation and improve the stability of the emulsions.

Pfg-NMR has been used frequently to measure the diffusion coefficient of different materials and chemicals (Ghi, Hill, & Whittaker, 2002). However, nowadays, NMR has gained more popularity in food applications due to its nondestructive nature (Voda & Van Duynhoven, 2009). NMR is very useful to study the physical and chemical properties of foods (Ablett, 1992). Despite the fact that NMR is less sensitive towards components present at a very low concentration, such as colorants and flavours, compared to other spectroscopic methods, it is still a very suitable method for food systems (Ablett, 1992). In food applications, NMR has been utilized in a wide range of studies, for example a study on the adsorption of caseinate in triacylglycerol-in water emulsions (Mine, 1997), droplet size measurement in emulsions (Métais & Mariette, 2003; Vermeir, et al., 2016), and electrostatic interaction between lysolecithin and chitosan in emulsions (Kwamman, Mahisanunt, Matsukawa, & Klinkesorn, 2016). In the field of milk proteins, pfg-NMR has been used to study the diffusion of WPI and the gel structure of WPI (Croguennoc, Nicolai, Kuil, & Hollander, 2001; Le Bon, Nicolai, Kuil, & Hollander, 1999). Nevertheless, to our knowledge, there is limited research which utilizes NMR as a means to study protein-polysaccharide conjugates. By determining the diffusion coefficient of protein-polysaccharide mixtures, native protein, and native polysaccharides using NMR, it became possible to confirm the formation of protein-polysaccharide conjugates and to quantify the degree of interaction between proteins and polysaccharides. Weinbreck, Rollema, Tromp, and de Kruif (2004) successfully studied the formation of WPI-gum arabic coacervates formed via electrostatic

interaction by evaluating the diffusivity of WPI and gum arabic. In NMR, compounds with a different molecular weight have different diffusion behaviour properties and these properties are altered upon interaction with other compounds. As interaction between proteins and polysaccharides results in a new compound with a higher molecular weight, a change in the average diffusion coefficient as well as in the distribution width will be observed. In previous studies, chemical analyses using reagent such as TNBS (Jimenez-Castano, et al., 2007; Setiowati, et al., 2016) and OPA (Wooster, et al., 2006) were performed to determine the degree of interaction between proteins and polysaccharides by determining the amount of available amino groups in the conjugates. These methods are sensitive but labour intensive and time consuming. NMR, on the other hand, has as good sensitivity but is less labour intensive.

In this study, pfg-NMR was used to investigate the interaction of WPI and LMP as influenced by pH, duration of the dry heat treatment (incubation time), and the concentration of LMP present in the conjugates. The diffusion coefficient and the distribution width of the WPI and LMP as influenced by these factors were reported. Furthermore, the amount of free WPI and LMP-bound WPI in the conjugates was determined.

5.2 Materials and Methods

5.2.1 Materials

The WPI was purchased from Davisco Foods International Inc. (Le Sueur, MN, USA). Protein analysis revealed that the WPI contained approximately 97.7% protein, whereby 85% of the protein is β-lactoglobulin (Van der Meeren, et al., 2005). Low methoxyl pectin (LMP) (UnipectinOB700) was obtained from Cargill (Ghent, Belgium) and contained 89.6% of dry matter. Deuterium oxide (D_2O > 99.8%) was purchased from Armar Chemicals (Switzerland).

5.2.2 Methods

5.2.2.1 Conjugate preparation

WPI-LMP conjugates were prepared following the method described in section 2.2.2.

5.2.2.2 High Resolution NMR

High-resolution pulsed field gradient (pfg) NMR diffusion analysis was performed with a Bruker Avance III spectrometer operating at a ^{1}H frequency of 500.13 MHz and equipped with a 5 mm DIFF30 gradient probe with a maximum gradient strength of 18 T/m. Pfg-NMR experiments were performed at 25 ^{o}C using a monopolar (single) stimulated echo pulse sequence. The samples (650 µL) were filled in 5 mm diameter glass NMR tubes (Armar Chemicals, Switzerland) and were measured upon varying the gradient strength up to 12 T/m, while keeping the gradient duration (δ) constant at 1 ms and the diffusion delay (Δ) fixed at 100 ms. The samples were prepared by dissolving the powder in 10 mM Na phosphate buffer (pH 6.5 and 7.2) and 5 mM Na acetate buffer (pH 5.5 and 5.0) containing 5 mM of sodium acetate (in D_2O) to obtain a protein and pectin concentration of 20 mg/mL and 10 mg/mL, respectively.

The obtained experimental echo attenuation ratio (I/Io) with up to 95% decay as a function of gradient strength was then fitted by Eq. 5.1a and Matlab 7.5.0.342 (R2007b) software (The Mathworks). Hereby, a mass-weighted lognormal distribution of diffusion coefficients was assumed.

$$\left(\frac{I}{I_0}\right)_{expt} = \int_0^{\infty} P_v(D_i) \cdot \frac{I}{I_0}(D_i) \cdot \, dD_i \qquad \text{(Equation 5.1a)}$$

$$\frac{I}{I_0}(D_i) = \exp\left(-D_i \cdot \gamma^2 \cdot G^2 \cdot \delta^2 \cdot \left(\Delta - \frac{\delta}{3}\right)\right) \qquad \text{(Equation 5.1b)}$$

$$P_v(D_i) = \frac{1}{\sqrt{2\pi} \cdot D_i \cdot \ln \sigma_g} \cdot \exp\left(-\frac{\left(ln(D_i) - ln\left(D_g\right)\right)^2}{2 \cdot \left(\ln \sigma_g\right)^2}\right) \qquad \text{(Equation 5.1c)}$$

$$D_a = D_g \cdot \exp\left(\frac{\left(\ln \sigma_g\right)^2}{2}\right) \qquad \text{(Equation 5.1d)}$$

$$\sigma_a = \sqrt{{D_a}^2 \cdot \left(exp\left(\left(\ln \sigma_g\right)^2\right) - 1\right)} \qquad \text{(Equation 5.1e)}$$

Protein and pectin samples may be characterized by a molecular mass range and hence, a polydisperse population of diffusion coefficients with a certain probability P_v. In our calculation, P_v was assumed to follow a lognormal mass-weighted diffusion coefficient distribution (Eq. 5.1c). The geometric mean diffusion coefficient (D_g) and geometric standard deviation (σ_g) were converted to the arithmetic mean diffusion coefficient (D_a) and arithmetic standard deviation (σ_a) of the lognormal mass-weighted diffusion coefficient distribution using Eq. 5.1d and Eq. 5.1e.

The degree of molecular interaction can be evaluated upon decomposing the WPI diffusion signal obtained from the WPI-LMP conjugate sample $\left[\frac{I}{I_0}\right]_{WPI,conjug.}$ into a free WPI fraction (i.e. the experimentally obtained non-conjugated WPI signal $\left[\frac{I}{I_0}\right]_{WPI,free}$) and a bound WPI fraction. The bound fraction can be determined upon estimating the diffusion signal of the reacted WPI $\left[\frac{I}{I_0}\right]_{WPI,bound}$ using Eq. 5.2 and Matlab 7.5.0.342 (R2007b software, The Mathworks). As the molar mass of pectin is much larger as compared to WPI, the molar mass of the conjugate is mostly determined by the pectin. Hence, the bound fraction can be determined assuming the experimentally obtained LMP diffusion signal to be a good approximation of the LMP-bound WPI diffusion signal as written down in Eq. 5.3 using the Solver add-in (Microsoft Excel 2010). For WPI-LMP conjugates, the diffusion signal of dry heated WPI which had the same incubation time as the WPI-LMP conjugates was used as the diffusion signal of the free WPI. Thus signal decomposition of a 2:1 conjugate Day 8 will be performed using the diffusion signal of dry heated WPI (1:0) Day 8. The WPI and LMP signal recorded at 0.6-1 ppm and 3.5-4.0 ppm were used in this calculation, respectively.

$$\left[\frac{I}{I_0}\right]_{WPI,conjug.} = \varphi_{free} \cdot \left[\frac{I}{I_0}\right]_{WPI,free} + \varphi_{bound} \cdot \left[\frac{I}{I_0}\right]_{WPI,bound} \quad \text{(Equation 5.2)}$$

$$\left[\frac{I}{I_0}\right]_{WPI,conjug.} = (1 - \varphi_{bound}) \cdot \left[\frac{I}{I_0}\right]_{WPI,free} + \varphi_{bound} \cdot \left[\frac{I}{I_0}\right]_{LMP,conjug.} \quad \text{(Equation 5.3)}$$

5.2.2.3 Emulsion preparation

10% oil in water emulsions stabilized by 0.5% WPI and 0.5% WPI-LMP mixtures (without dry heat treatment) were prepared using the method described in section 3.2.3.

5.2.2.4 Electrophoretic mobility measurement

The electrophoretic mobility of the emulsions was measured using a Zetasizer 2c (Malvern Ltd, UK) as explained in section 3.2.9.

5.3 Results

5.3.1 Diffusion coefficient of native WPI and LMP

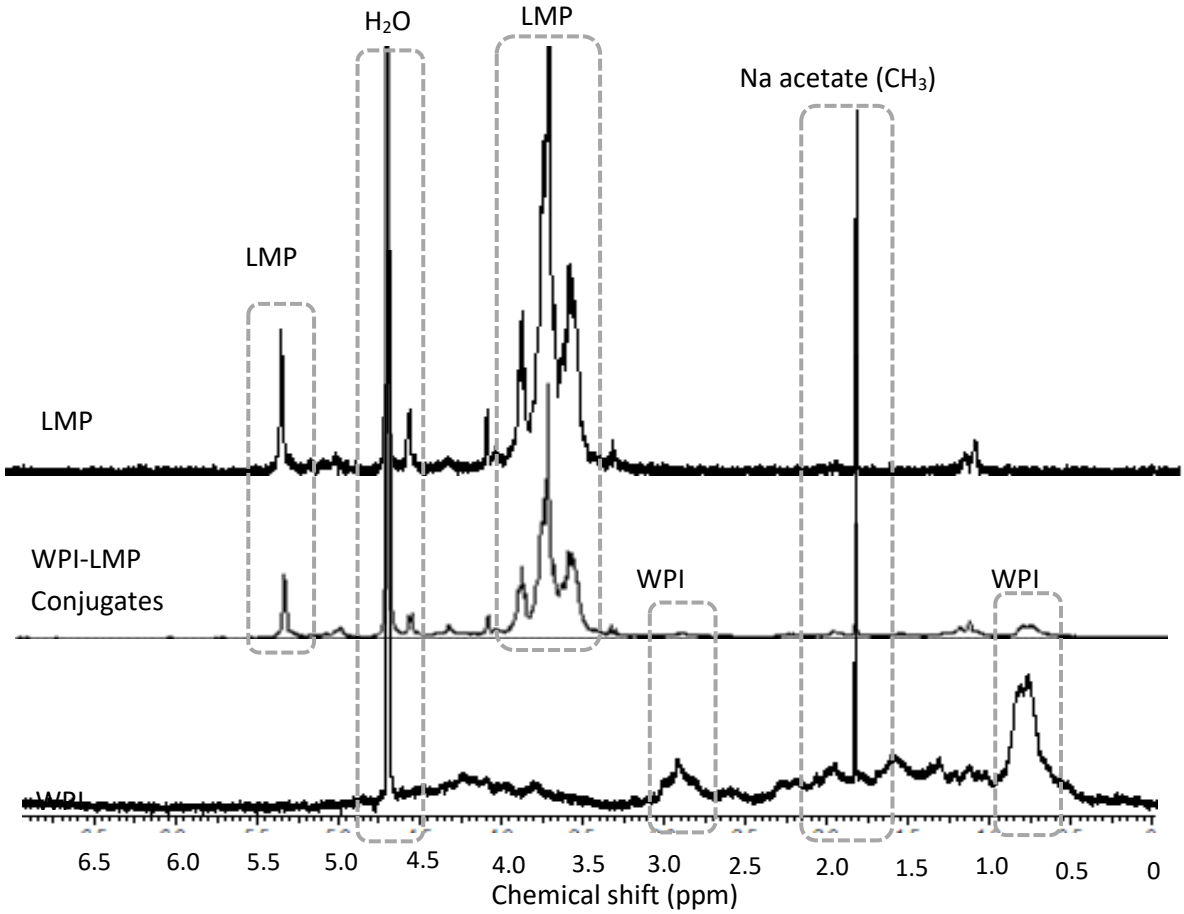


Figure 5.1. 1D ^{1}H spectrum of Native WPI, LMP, and WPI-LMP conjugates (at 2:1 WPI:LMP ratio) recorded at 25^{o}C.

Pfg-NMR with a high gradient probe was used to determine the diffusion behaviour of WPI and LMP. A high gradient probe was needed due to the high molecular weight of WPI and LMP. From the NMR measurement, 1D spectra of native WPI and LMP were obtained (Fig. 5.1). By comparing the spectra of WPI and LMP, it was found that the signal observed at 0.6-1 ppm and 3.7-3.1 belonged to WPI, while the signal at 3.3-4.2 ppm and 5.2-5.5 ppm belonged to LMP. Thus, in a system containing WPI and LMP, it was possible to distinguish the diffusion coefficient of each biopolymer. The arithmetic mean diffusion coefficient (D_a) and arithmetic standard deviation (σ_a) of WPI and LMP can be found in Table 5.1.

The results presented in Table 5.1 and Figure 5.2 indicate that WPI ($7 \cdot 10^{-11}$ m2/s) diffuses much faster than LMP ($5 \cdot 10^{-11}$ m^2/s). Furthermore, it was obvious that the arithmetic standard deviation, which represents the distribution width, of LMP was almost ten times bigger than the arithmetic standard deviation of WPI. WPI mainly consists of β-lactoglobulin which has a molecular weight of 18 kDa (monomer) with a narrow range of molecular weights. On the other hand, LMP has a bigger molecular weight of approximately several hundred kDa (De Vries, et al., 1982) and a broader range of molecular weights. While the former explains the slow diffusion of LMP compared to WPI, the latter explains the broad diffusion coefficient distribution exhibited by LMP (Fig. 5.2). In contrast, WPI had a much narrower distribution width due to its narrow molecular weight distribution since it mainly consists of β-lactoglobulin.

In order to evaluate the influence of dry heat treatment on WPI, freeze dried WPI was dry heat treated up to 16 days. Previously, SDS-PAGE analysis revealed that a limited protein polymerization occurred in dry heat treated WPI (Setiowati, et al., 2016). Table 5.2 revealed that upon dry heat treatment of WPI, the diffusion coefficient of WPI did not decrease significantly. Polymerization of protein leads to the formation of molecules with higher molecular weight (Jiménez-Castaño, Villamiel, et al., 2005) which diffuse slower. It can be seen in Table 5.2 that the distribution width of WPI became two times broader after 8 days of dry heat treatment. This might be due to polymerization of some of the proteins molecules in WPI.

Hereinafter, the discussion of the results is divided into 3 parts, i.e.: influence of pH, incubation time, and LMP concentration (WPI to LMP ratio).

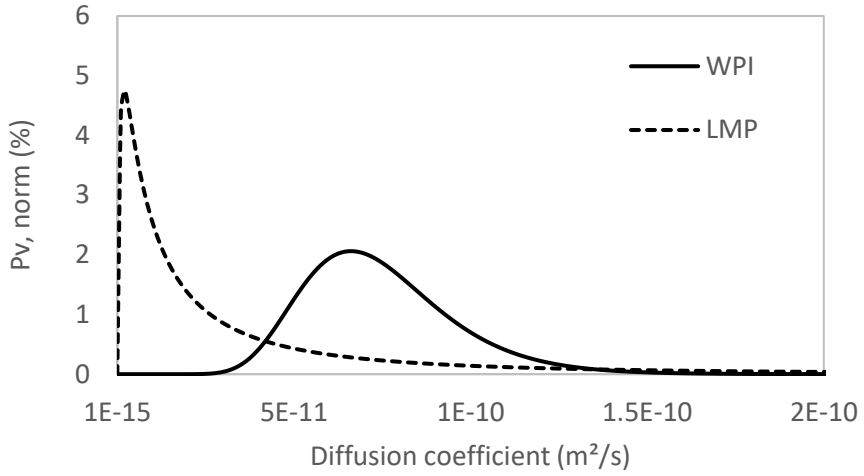
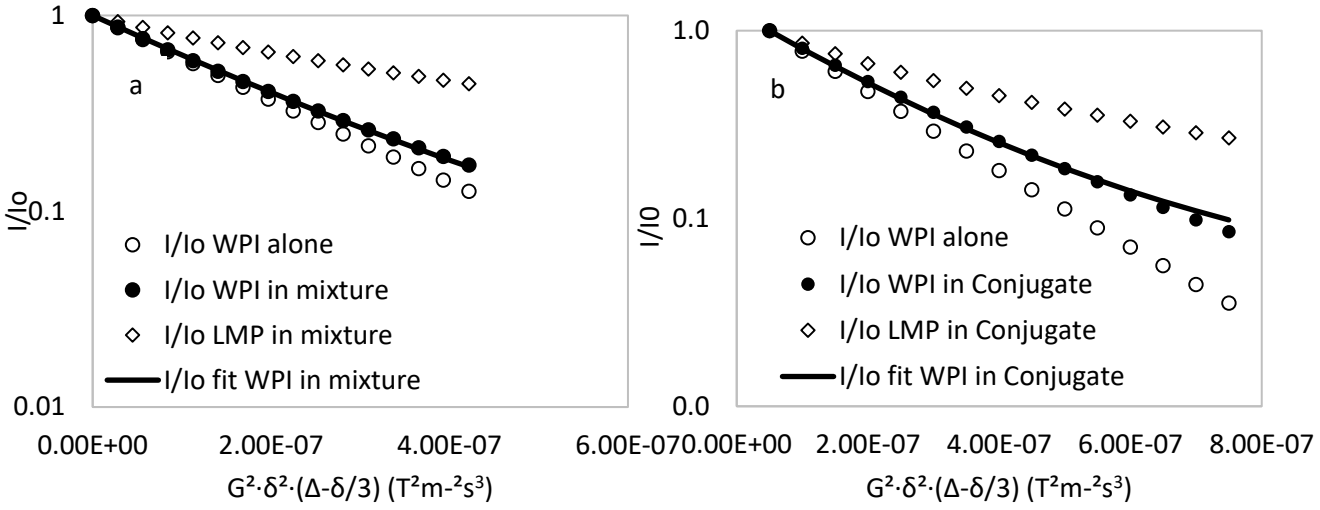


Figure 5.2. Normalized probability density function of the lognormal volume-weighted diffusion coefficient distributions of the WPI (0.6-1 ppm) and LMP (3.5-4.0 ppm).

Table 5.1 Arithmetic mean diffusion coefficient (D_a) and arithmetic standard deviation (σ_a) value of WPI, LMP, and WPI-LMP conjugates dry heat treated for 0-16 days obtained upon fitting Eq. 5.1 to the diffusion signal of the WPI and LMP contributions in the non-conjugated and conjugated samples.

Sample	pH	WPI 0.6-1 ppm		Prot 2.7-3.1 ppm		LMP 3.5-4.0 ppm		LMP 5.31-5.37 ppm	
		D_a (m^2/s)	σ_a (m^2/s)	D_a (m^2/s)	σ_a (m^2/s)	D_a (m^2/s)	σ_a (m^2/s)	D_a (m^2/s)	σ_a (m^2/s)
WPI	7.2	7.13E-11	1.42E-11	6.85E-11	5.02E-12				
LMP	7.2					5.49E-11	1.61E-10	5.24E-11	1.15E-10
2:1 Day 0	7.2	7.05E-11	3.52E-11	6.61E-11	2.17E-11	5.45E-11	5.92E-11	3.88E-11	3.17E-11
2:1 Day 1	7.2	6.51E-11	3.14E-11	6.75E-11	2.65E-11	5.80E-11	7.53E-11	4.08E-11	4.95E-11
2:1 Day 2	7.2	6.42E-11	3.54E-11	6.27E-11	2.98E-11	4.94E-11	5.73E-11	3.91E-11	4.09E-11
2:1 Day 8	7.2	6.27E-11	4.14E-11	6.52E-11	4.15E-11	4.70E-11	5.72E-11	3.46E-11	3.78E-11
2:1 Day 16	7.2	5.97E-11	3.54E-11	5.85E-11	3.54E-11	4.75E-11	5.28E-11	5.55E-11	8.63E-11
4:1 Day 8	7.2	7.15E-11	3.63E-11	7.08E-11	3.36E-11	6.41E-11	5.96E-11	5.37E-11	4.52E-11
1:1 Day 16	6.5-6.8	8.8E-11	1.41E-10			5.4E-11	1.5E-10	3.3E-11	8.8E-11
2:1 Day 0	5.5	5.95E-11	3.37E-11	6.00E-11	3.17E-11	5.19E-11	8.12E-11	4.25E-11	4.93E-11
2:1 Day 0	5	5.67E-11	3.70E-11	7.50E-11	5.32E-11	6.55E-11	1.37E-10	4.13E-11	6.39E-11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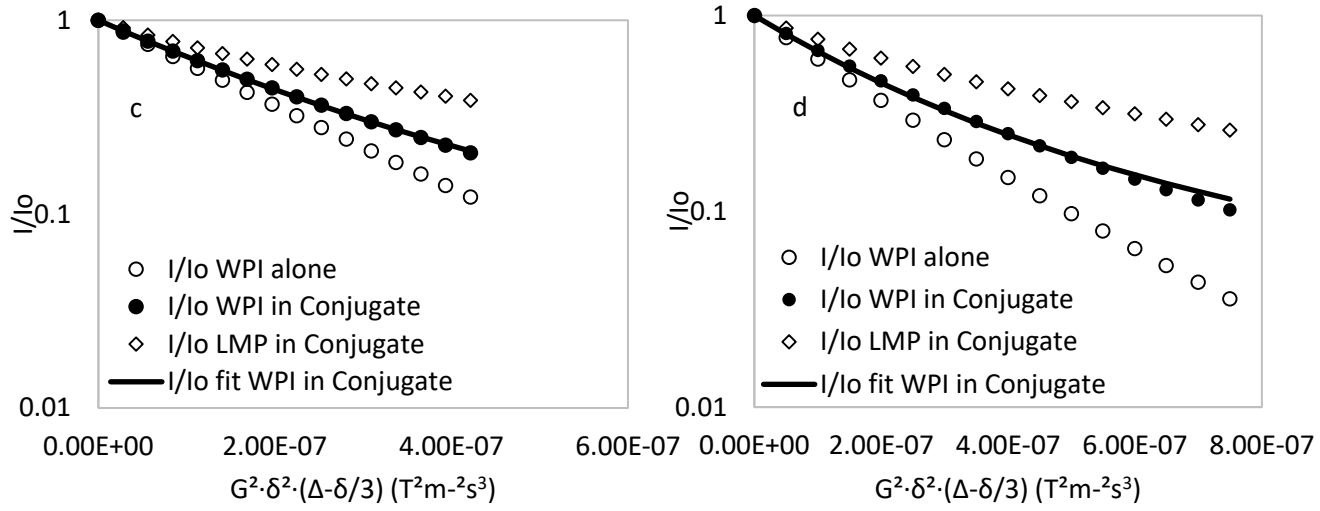
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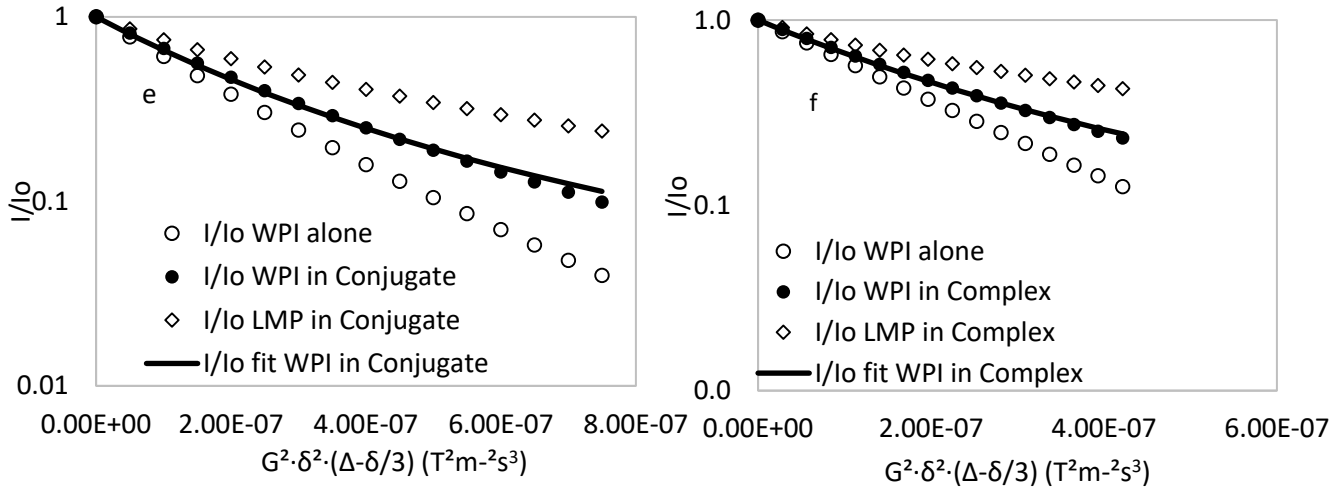
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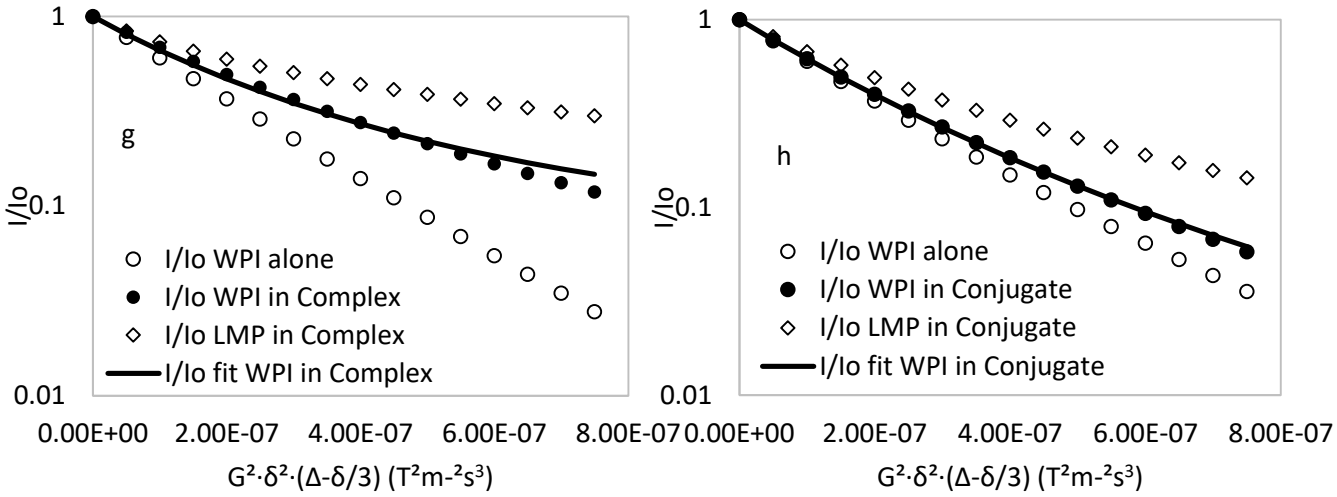


Figure 5.3 Decomposition of WPI diffusion signal in the presence of LMP into the calculated protein diffusion signal without pectin (lyophilized/dry heat treated WPI only (1:0)) and the calculated pectin signal in WPI-LMP conjugates of ratio 2:1 as a function of incubation time (measured at pH 7.2): a) Day 0, b) Day 1, c) Day 2, d) Day 8, e) Day 16, WPI-LMP mixture ratio 2:1 f) pH 5.5, g) pH 5.0, and h) WPI-LMP conjugates ratio 4:1 Day 8.

Table 5.2. Arithmetic mean diffusion coefficient (D_a) and arithmetic standard deviation (σ_α) value of WPI, which has been dry heat treated for 0-16 days obtained upon fitting Eq. 5.1 to the diffusion signal of the WPI contributions in the samples.

Sample	WPI 0.6-1 ppm		WPI 2.7-3.1 ppm	
	D_a (m^2/s)	σ_α (m^2/s)	D_a (m^2/s)	σ_α (m^2/s)
1:0 Day 0 (freeze dried)	7.13E-11	1.42E-11	6.85E-11	5.02E-12
1:0 Day 1	7.11E-11	1.36E-11	7.48E-11	2.07E-11
1:0 Day 2	7.19E-11	1.28E-11	7.39E-11	2.10E-11
1:0 Day 8	7.39E-11	2.56E-11	8.05E-11	3.53E-11
1:0 Day 16	7.16E-11	2.52E-11	6.95E-11	2.01E-11

5.3.2 Influence of pH

It is expected that interaction between WPI and LMP results in a complex whose molecular weight is higher than that of WPI and LMP alone, which alters the diffusion properties of each component. Due to this, a decrease of the diffusion coefficient value is expected to take place upon interaction. This means that formation of WPI-LMP conjugates can be confirmed by comparing the diffusion coefficient of the WPI and LMP signal before and after

conjugate/complex formation. Furthermore, by decomposing the signal decay of the WPI in the conjugate into the WPI signal and the LMP, the fraction of free and LMP-bound WPI can be determined. A typical 1D ^{1}H spectrum of a WPI-LMP mixture obtained from NMR can be seen in Fig. 5.1.

In this section, the influence of pH on the diffusion coefficient WPI/LMP in a mixture containing WPI and LMP is discussed. For this purpose, mixtures of WPI and LMP with ratio of 2:1 were prepared at pH close to the IEP of WPI (5.0 and 5.5) and at relatively neutral pH (7.2). It is indeed well known that, the electrostatic interaction between WPI and LMP is governed by pH. Fig. 5.4 shows that when the pH was decreased to around the IEP of WPI, the diffusion echo decay for both LMP and WPI in the mixture decreased less rapidly which indicated slower diffusion due to molecular interaction between WPI and LMP. At pH 5.0, this effect was more pronounced than that at pH 5.5. Accordingly, it was expected that there was more WPI bound to LMP at pH 5.0 than at pH 5.5.

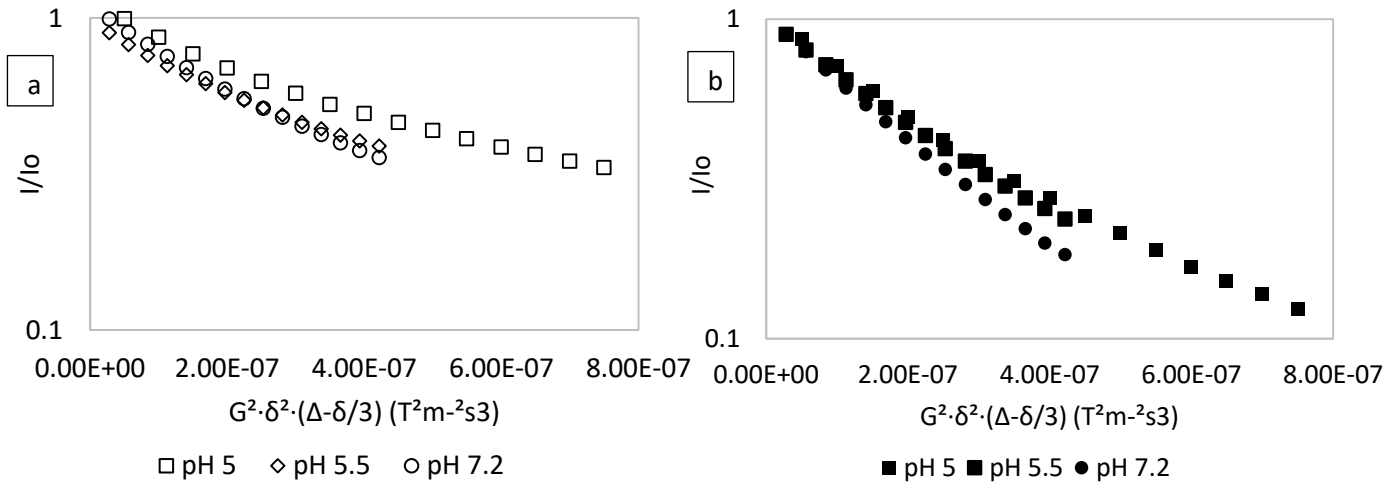


Figure 5.4. Diffusion echo decay of the a) LMP signal (3.5-4.0 ppm) and b) WPI signal (0.6-1.0 ppm) in WPI-LMP mixtures (without dry heat treatment, ratio 2:1, Day 0) as a function of pH.

The diffusion coefficient of WPI and LMP in the mixtures (2:1 Day 0) prepared at pH 7.2, 5.5 and 5.0 can be found in Table 5.1. The diffusion coefficient of the WPI (0.6-1.0 ppm) in the mixtures were $7.05 \cdot 10^{-11}$ m^2/s, $5.95 \cdot 10^{-11}$ m^2/s, and $5.67 \cdot 10^{-11}$ m^2/s at pH 7.2, 5.5, and 5.0 respectively (Table 5.1). These values were smaller compared to that of native WPI ($7.13 \cdot 10^{-11}$ m^2/s). Generally for the WPI in the presence of LMP, the diffusion coefficient decreased as the pH lowered. The distribution width of WPI increased from $1.42 \cdot 10^{-11}$ m^2/s (native WPI) to

± $3.5 \cdot 10^{-11}$ m^2/s in the presence of LMP. On the other hand, the diffusion coefficient of the LMP signal (3.5-4.0 ppm) at pH 7.2, 5.5, and 5.0 was $5.45 \cdot 10^{-11}$ m^2/s, $5.19 \cdot 10^{-11}$ m^2/s, and $6.55 \cdot 10^{-11} m^2/s$ respectively. Strong electrostatic interaction between biopolymers reduces the diffusion coefficient of biopolymers (Weinbreck, et al., 2004). Hence, it was predicted that the diffusion coefficient of LMP at pH 5.0 would be the smallest. However, it was found that at pH 5.0 the diffusion coefficient of LMP was higher than that at pH 7.2 and 5.5. Nevertheless, It has to be noted that the LMP peak at pH 5.0 exhibited a broader diffusion coefficient distribution ($1.37 \cdot 10^{-10}$ m^2/s) than at pH 7.2 ($5.92 \cdot 10^{-11}$ m^2/s) and 5.5 ($8.12 \cdot 10^{-11}$ m^2/s) (Table 5.1). For a constant geometric mean, the arithmetic mean increases with increasing distribution width. This phenomenon can also be observed in Fig. 5.5; the WPI and LMP signal in the mixture at low pH had a broader distribution width.

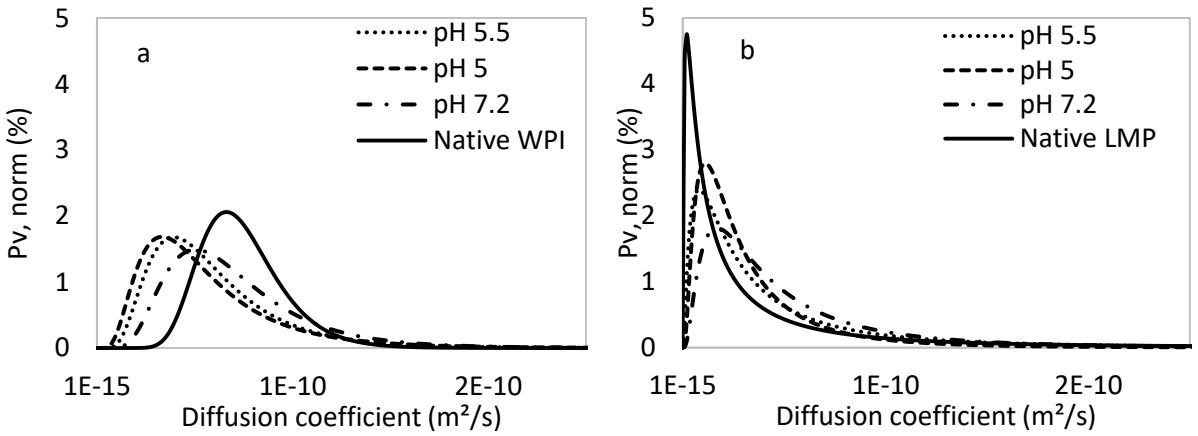


Figure 5.5. The normalized lognormal volume-weighted probability density function of the diffusion coefficient of the lognormal volume-weighted distributions of the native WPI (0.6-1.0 ppm) (a) and LMP (3.5-4.0 ppm) (b) in a mixture of WPI-LMP prepared at pH 7.2, 5.5, and 5.0.

From the decomposition of the WPI signal in the mixture into the calculated WPI diffusion signal without LMP (lyophilized/dry heat treated WPI only) and the calculated pectin signal with LMP (WPI-LMP conjugates/ WPI-LMP complex) (Figure 5.3 a, f, and g), the fraction of unreacted WPI or free WPI in the mixture was calculated. At pH 7.2, there was approximately 13% of WPI bound to LMP and 87% of free WPI. At pH 5.5 and 5.0, the amount of LMP-bound WPI increased, which was expected. There was 39% of WPI bound to LMP at pH 5.5. This amount increased to 44% as the pH was decreased to 5. This result explains the trend

observed in the diffusion coefficient of WPI previously mentioned. In addition, it can be noticed in Figure 5.3 a, f, and g that the diffusion signal of WPI ($I/Io_{WPI\ complex}$) shifted further to the right towards the diffusion signal of LMP as more WPI bound to LMP.

WPI has an IEP at around pH 4.8-5 and carries a net negative charge above its IEP and positive charge below its IEP (Demetriades, et al., 1997a). The further the pH of the proteins from the IEP, the bigger the net charge of the proteins is (Fig. 5.6). Therefore, WPI at pH 7.2 was expected to be more negatively charged than that at pH 5.5 and pH 5.0 which was close to the IEP of WPI. Figure 5.6 shows the electrophoretic mobility profile of emulsions stabilized by WPI only and WPI-LMP mixture. The figure indicates that at pH 5.0-5.5, the EM of emulsions stabilized by a WPI-LMP mixture was more negative than that stabilized by WPI only. This was due to the presence of LMP at the surface of oil droplets via electrostatic interaction with positive patches on WPI. At pH higher than 6 the EM of emulsions stabilized by WPI only and WPI-LMP was found to be comparable. Electrostatic attraction takes place when two biopolymers carry an opposite charge (Ye, 2008). LMP is an anionic polysaccharides and is negatively charged. Therefore, at a higher pH, electrostatic attraction between WPI and LMP is not favourable due to charge repulsion since both biopolymers carry a negative charge (Gu, et al., 2005). WPI was also negatively charged at pH 5.5 and 5.0. However, the net charge is lower. Moreover, the small net negative charge follows from a surplus of negative charges over positive charges. Hence, at pH above the protein's IEP, a weak electrostatic attraction can take place between anionic pectin and the positively charged regions of the WPI (Girard, Turgeon, & Gauthier, 2002; Imeson, Ledward, & Mitchell, 1977). The same authors also mentioned that hydrogen bonds are also involved in the interaction between WPI and pectin at a pH slightly above the IEP of the protein, albeit to a lesser extent. This might also explain the weak interaction between WPI and LMP that took place at pH 7.2.

These results also implied that measurement of the WPI-LMP conjugates should be performed at relatively neutral pH (7.0) to minimize electrostatic interaction between WPI and LMP which might cause an overestimation of the result.

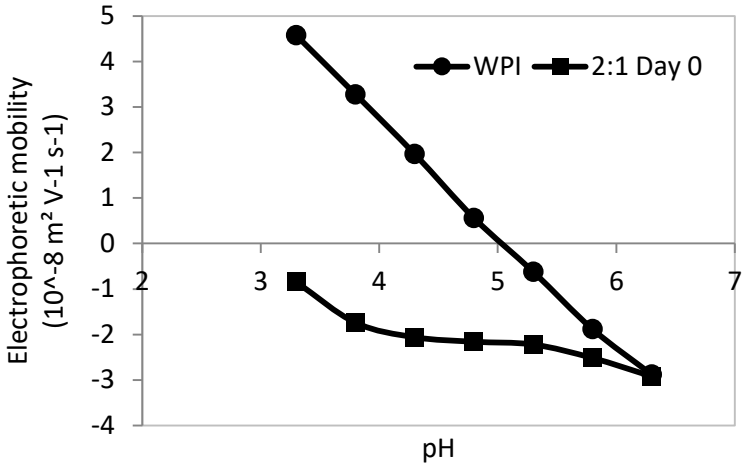


Figure 5.6 Electrophoretic mobility profile of emulsions stabilized by native WPI and by WPI-LMP mixtures (without dry heat treatment).

Table 5.3 Percentage of free WPI in the samples obtain from the decomposition of the echo decay of WPI diffusion signal in the mixture.

WPI:LMP ratio	Dry heat incubation (days)	pH	Free WPI (%)
2:1	0	5.5	61
	0	5.0	56
2:1	0	7.2	87
	1	7.2	73
	2	7.2	66
	8	7.2	64
	16	7.2	63
4:1	8	7.2	75
1:1	16	6.5-6.8	52

5.3.3 Influence of incubation time

The interaction between WPI and LMP as influenced by incubation time was determined at pH 7.2. SDS-PAGE analysis performed in a previous study has showed that dry heat treatment of WPI-LMP mixtures resulted in the formation of high molecular weight compounds (WPI-LMP conjugates) (Setiowati, et al., 2016). This study was performed to investigate the possibility of NMR to be used as a tool to confirm the formation of WPI-LMP conjugates and quantify the formation of conjugates over incubation time. The results were also compared with those obtained with TNBS analysis performed in a previous study. Since WPI-LMP

conjugates had a higher molecular weight, WPI was expected to have a smaller diffusion coefficient than that of WPI alone due to the presence of covalently linked LMP. In this study, WPI-LMP conjugates with WPI to LMP ratio of 2:1 incubated or dry heat treated for 0, 1, 2, 8, and 16 days were prepared.

Figure 5.7 below shows the diffusion echo decay of the LMP and WPI signal in the conjugates. As it can be observed, the longer the dry heat treatment time, the more the graphs shifted upward. However, it can be seen that the diffusion echo decay of the WPI in WPI-LMP conjugates obtained after 8 or 16 days or dry heat treatment almost coincided. The observed upward shift was due to the increase of the molecular weight of WPI/LMP caused by the conjugation of WPI and LMP. Upon dry heat treatment, the amino groups of WPI are covalently linked to the carboxyl groups of LMP through a Maillard type reaction (Dickinson, 2008). A longer incubation time was expected to result in a higher degree of interaction between WPI and LMP leading to the reduction of the free WPI fraction in the system.

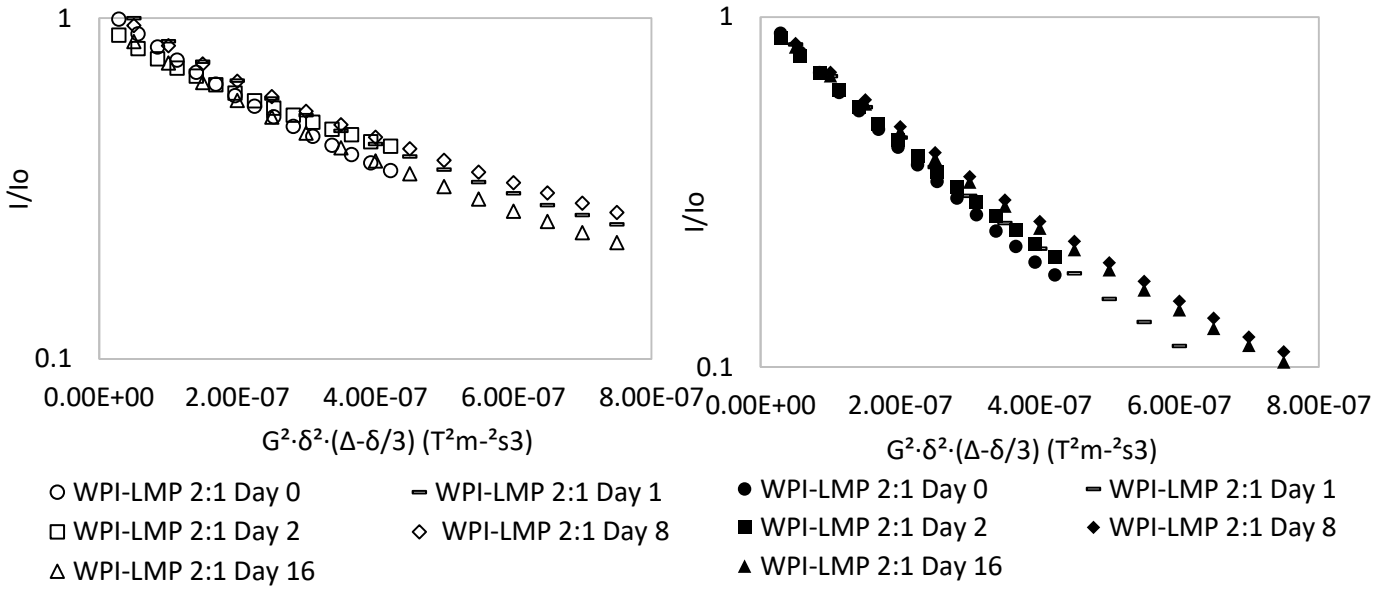


Figure 5.7. Diffusion echo decay of the LMP signal (3.5-4.0 ppm; open symbols) and WPI signal (0.6-1.0 ppm; filled symbols) in WPI-LMP conjugates prepared at pH 7.2 as a function of dry heat incubation time.

Table 5.1 reveals that dry heat treatment for one day slightly decreased the diffusion coefficient of WPI from $7.05 \cdot 10^{-11}$ m^2/s (Day 0) to $6.51 \cdot 10^{-11}$ m^2/s. Further incubation of the conjugate resulted in further reduction of the WPI diffusion coefficient to $6.42 \cdot 10^{-11}$ m^2/s after 2 days of dry heat treatment, $6.27 \cdot 10^{-11}$ m^2/s after 8 days of dry heat treatment, and $5.97 \cdot 10^{-}$

^{11} m^2/s after 16 days of dry heat treatment. A change in the distribution width was also observed (Table 5.1). Compared to the distribution width of native WPI, the WPI signal in conjugates had a broader distribution width (Fig. 5.8). As for LMP, the trend was similar: the diffusion coefficient of LMP decreased as the incubation time was prolonged. The LMP signal had a diffusion coefficient of $5.45 \cdot 10^{-11}$ m^2/s, $5.80 \cdot 10^{-11}$ m^2/s, $4.94 \cdot 10^{-11}$ m^2/s, $4.70 \cdot 10^{-11}$ m^2/s, and $4.75 \cdot 10^{-11}$ m^2/s after dry heat treatment for 0, 1, 2, 8 and 16 days, respectively.

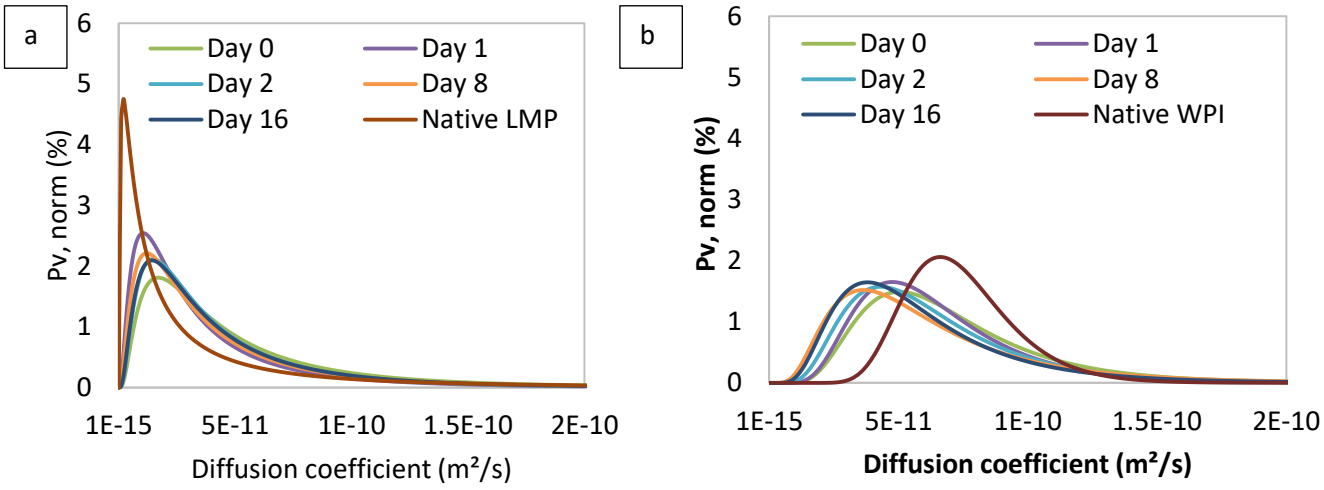


Figure 5.8. Normalized lognormal volume-weighted probability density function of the diffusion coefficient of LMP (3.5-4.0 ppm) (a) and WPI (0.6-1.0 ppm) (b) in WPI-LMP conjugates which were incubated for 0, 1, 2, 8, and 16 days.

Signal decomposition of the WPI signal in the conjugates into the WPI signal without and with LMP can be seen in Fig. 5.3 a, b, c, d, and e. From the calculation, it was found that in the unheated WPI-LMP mixture (Day 0), there was already 13% of WPI bound to LMP. SDS-PAGE analysis has shown that without dry heat treatment, there was no conjugate formation in WPI-LMP mixtures (Setiowati, et al., 2016). Hence, in this case, WPI-LMP complexes were probably formed due to weak interaction between WPI and LMP as explained in the previous section. From the calculation, it was observed that upon dry heat treatment, the percentage of free WPI decreased from 87% (Day 0) to 73%, 66%, 64%, and 63% after 1, 2, 8, and 16 days of dry heat treatment. Fig. 5.9 reveals that the WPI-LMP mixtures underwent rapid formation of conjugates in the first 2 days of dry heat treatment. Extension of the dry heat treatment for more than 2 days did not considerably increase the amount of conjugates formed. In our previous research, the degree of interaction between WPI and LMP was determined by measuring the availability of the primary amino group of WPI in the conjugates as a function

of incubation time using the TNBS method (Setiowati, et al., 2016). It was found that after 16 days of dry heat treatment, WPI-LMP conjugates with a ratio 2:1 had a degree of conjugation of approximately 15% which is lower than the value obtained with NMR. However, it has to be noted that β-lactoglobulin has 15 lysine residues. Thus, if one out of the 15 residues of the amino groups reacts (i.e.: 6.67% based on free amino groups), all WPI maybe bound to LMP (i.e.: 100% based on diffusion NMR). Furthermore the results also showed that after four days of dry heat treatment, the degree of interaction between WPI and LMP did not increase significantly which is in agreement with the trend observed in this study. The formation of milk protein-pectin conjugates with improved emulsifying properties has been reported to take several days ranging from 10 to 16 days (Einhorn-Stoll, et al., 2005; Neirynck, et al., 2004). Nevertheless, a recent study reported that one day of dry heat treatment was sufficient to produce WPI-LMP conjugates with improved emulsifying properties (Setiowati, et al., 2017). The NMR measurements in this study confirmed that WPI-LMP conjugates were already formed within 1 day of dry heat treatment.

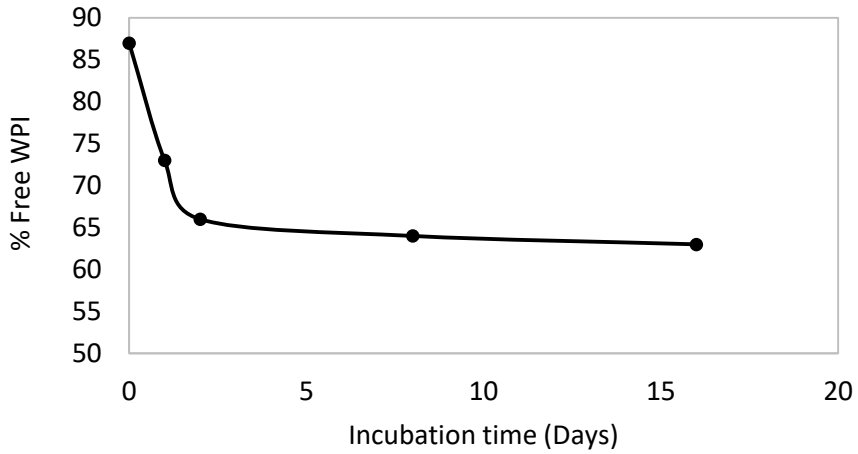


Figure 5.9 Fraction of free WPI found in the WPI-LMP conjugates (ratio 2:1) as a function of incubation time.

With the same WPI to LMP ratio, the amount of WPI bound to LMP obtained via dry heat treatment of WPI and LMP for 1-2 days was lower than that obtained via electrostatic interaction at pH 5.0 (Table 5.3). Nevertheless, the emulsifying activity and heat stability of WPI-LMP conjugates which was dry heat treated for 1-2 days was better than that obtained via electrostatic interaction at pH 5.0. Whereas the former was found to be heat stable in the presence of NaCl, the latter was highly unstable when NaCl was added (Setiowati, et al., 2017).

This result once again showed that the nature of interaction between WPI and LMP is highly important.

5.3.4 Influence of WPI to LMP ratio

The influence of the WPI to LMP ratio was also investigated. In this study, the concentration of the WPI was kept the same at all ratios, while the concentration of the LMP was varied. WPI-LMP conjugates with four different WPI to LMP ratios, namely ratio 1:0, 4:1, 2:1, and 1:1 were prepared. The conjugates with ratio of 1:0, 4:1, and 2:1 were incubated for 8 days, while conjugates with ratio 1:1 were incubated for 16 days. This comparison was made since an extension of the dry heat treatment time from 8 days to 16 days did not considerably increase the amount of conjugates. The conjugates were measured at pH 7.2 in a phosphate buffer containing 5 mM of sodium acetate, except for the conjugate with ratio 1:1. The latter was measured in D_20 containing 5 mM sodium acetate with a final pH of approximately 6.5-6.8.

The diffusion echo decay of the LMP and WPI signal as influenced by the WPI to LMP ratio can be found in Figure 5.10. In the case of LMP, the graphs shifted upward as the concentration of LMP was higher. The same trend was observed for WPI. It can be seen in Table 5.1 that the conjugates with ratio 1:0, 4:1, 2:1, and 1:1 had a diffusion coefficient of $7.45 \cdot 10^{-11}$ m^2/s, $7.15 \cdot 10^{-11}$ m^2/s, $6.27 \cdot 10^{-11}$ m^2/s, and $8.80 \cdot 10^{-11}$ m^2/s, respectively, when considering the WPI peak at 0.6-1.0 ppm. On the other hand, the distribution width of the conjugates were reported to be $2.56 \cdot 10^{-11}$ m^2/s, $3.63 \cdot 10^{-11}$ m^2/s, $4.14 \cdot 10^{-11}$ m^2/s, and $14.1 \cdot 10^{-11}$ m^2/s for the conjugates with ratio of 1:0, 4:1, 2:1, and 1:1, respectively. It was found that the diffusion coefficient of WPI decreased as the LMP concentration was increased from WPI to LMP ratio of 1:0 to 2:1. While it was expected that the diffusion coefficient of the WPI signal at ratio 1:1 would be the smallest, it was found that it had the biggest diffusion coefficient. Nevertheless, it has to be noted that the WPI signal at this ratio exhibited a much broader distribution width than the others (Fig. 5.11). Besides, Figure 5.11 clearly indicates that the distribution mode (i.e.: the diffusion coefficient corresponding to the maximum in the distribution curve) gradually decreased as the LMP concentration increased. Since native WPI had a very narrow distribution width due to its relatively uniform molecular weight, the presence of bound LMP would dramatically influence the diffusion coefficient and distribution width of WPI in conjugates. It was noticed that the more WPI became bound to LMP, the more it acquired a

similar diffusion behaviour as LMP molecules: LMP indeed has a broad range of molecular weights. Thus, the presence of covalently linked LMP in WPI molecules broadens the distribution width of WPI (Fig. 5.11). The results indicated that interaction between WPI and LMP did not only decrease the diffusion coefficient of WPI, but also broadened the distribution width. Nevertheless, when the distribution width becomes broader, it might also cause the calculated average diffusion coefficient to be bigger since at a constant geometric mean, the arithmetic mean increases with increasing distribution width.

The signal decomposition and calculation of the free WPI fraction in the conjugates confirmed the phenomena explained above (Table 5.3). After 8 days of dry heat treatment, there was 75% of unreacted WPI in the conjugates with ratio 4:1. By Increasing the proportion of LMP in the conjugates to WPI:LMP ratio of 2:1, the fraction of free WPI present in the conjugate decreased to 64%. Finally, at ratio 1:1 almost half of the WPI was bound to LMP (Table 5.3). Therefore, it can be concluded that increasing the concentration of LMP in the WPI-LMP conjugates resulted in a higher yield or higher degree of conjugation between WPI and LMP. It was suggested that, by increasing the concentration of LMP, the concentration of carbonyl groups available for conjugate formation increased. As a consequence, there were more WPI molecules that could react with LMP during dry heat treatment leading to the reduction of the amount of free WPI in the conjugates.

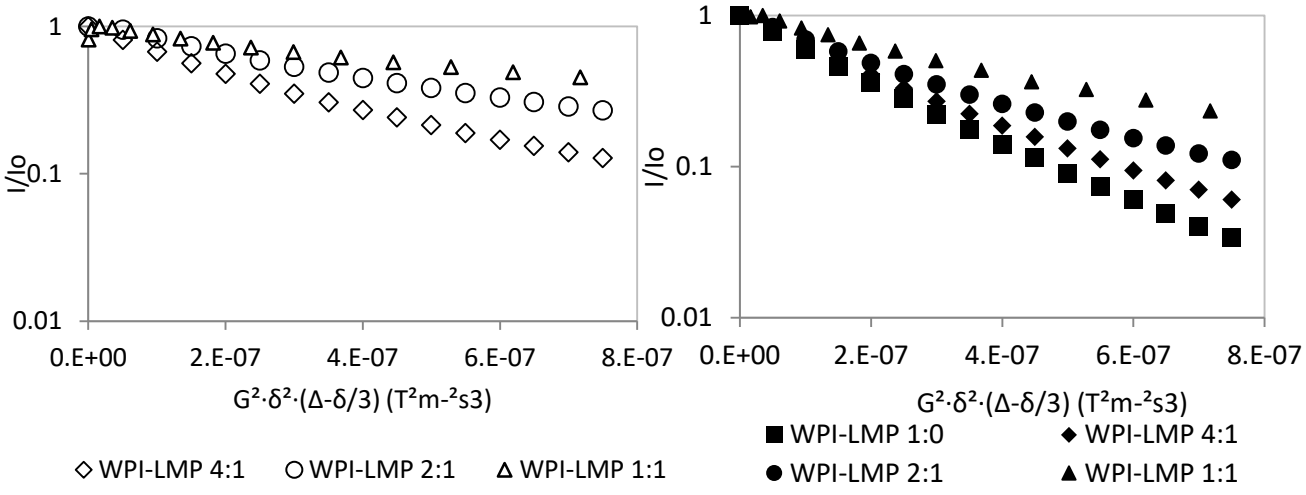


Figure 5.10. Diffusion echo decay of the LMP signal (3.5-4.0 ppm) and WPI signal (0.6-1.0 ppm) in WPI-LMP conjugates at ratio 1:0, 4:1, 2:1, and 1:1 prepared at pH 7.2 after 8 days (ratio 1:0, 2:1, and 4:1) or 16 days (ratio 1:1) of dry heat incubation time.

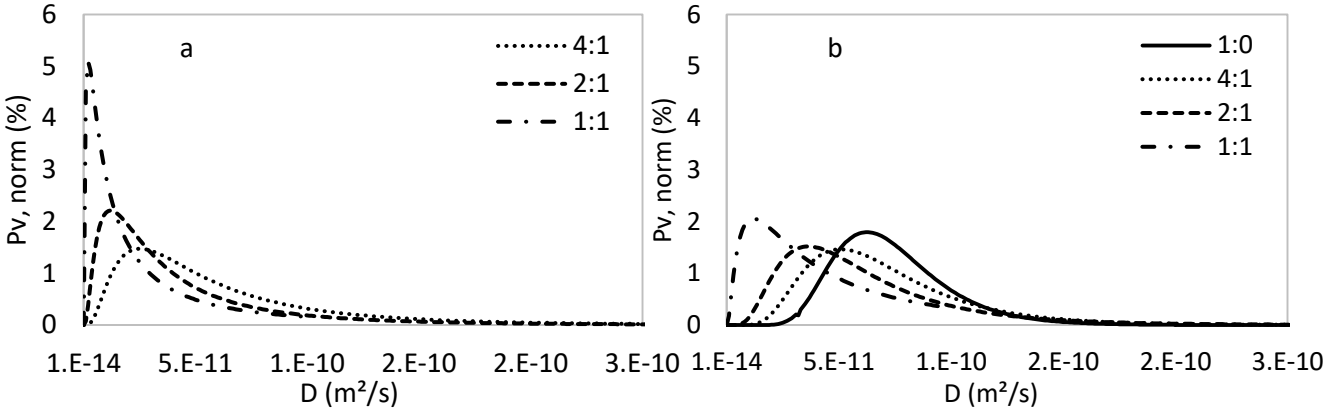


Figure 5.11. The normalized lognormal volume-weighted probability density function of the diffusion coefficient of LMP (3.5-4.0 ppm) (a) and WPI (0.6-1.0 ppm) (b) signal in WPI-LMP conjugates with ratio of 1:0, 4:1, 2:1, and 1:1.

Based on the results, it seems that the formation of conjugates was influenced more by the ratio of WPI to LMP than by the duration of the dry heat treatment. WPI-LMP conjugates with ratio 1:1 which was incubated for 16 days had a higher degree of interaction between WPI and LMP (±50%) than that with ratio 2:1 even after 16 days of dry heat treatment. Therefore, in order to obtain a high yield or a high amount of conjugated WPI-LMP it is recommended to increase the concentration of LMP. Nevertheless, it has been found that as long as the WPI-LMP is dry heat treated, a lower LMP concentration (ratio 4:1 and 2:1) is enough to produce WPI-LMP conjugates with better emulsifying activity and heat stability than native WPI and WPI-LMP mixtures (Setiowati, et al., 2017). This study also revealed that increasing the concentration of LMP in WPI-LMP conjugates resulted in an emulsion with smaller droplet size, but the heat stability was comparable at all LMP concentrations. The ability of WPI-LMP conjugates with higher LMP concentration to produce emulsions with smaller droplet size might be related to the higher degree of interaction between WPI and LMP found in WPI-LMP conjugates with higher LMP concentration. Hereby, as far as heat stability is concerned, a lower concentration of LMP is already sufficient to obtain WPI-LMP conjugates with superior heat stability. However, a higher concentration of LMP seems to be needed to obtain emulsions with smaller droplet size.

The results of this study indicate that NMR is a promising method to study protein and polysaccharide interaction. The method is user-friendly and sample preparation is relatively

short. A lot of useful information can be extracted from the NMR measurements, such as the molecular structure, diffusion coefficient and distribution width, which are related to the molecular weight distribution of the targeted compound, and degree of interaction between two compounds. Compared to the results obtained from TNBS measurement (Setiowati, et al., 2016), the degree of interaction between WPI and LMP obtained using NMR was higher. This could be due to the fact that in TNBS, the measurement was performed by quantifying the amount of free amino groups present in the conjugates after dry heat treatment. Proteins contain several amino groups per molecule: as an example, β-lactoglobulin, the most abundant whey protein, contains 15 lysine residues. Hence, a major part of the whey proteins can become conjugated to polysaccharides, despite of the fact that only a small reduction in free amino group content was observed. On the other hand, NMR measures the proteins by considering them as whole molecules. Despite of showing a different magnitude on the degree of interaction, the general trend obtained from the NMR measurements was comparable with that obtained from TNBS measurement. The pH of the sample was also found to be crucial for the NMR measurement. Sample preparation for NMR analysis should take into account the influence of pH on the interaction of the targeted compounds.

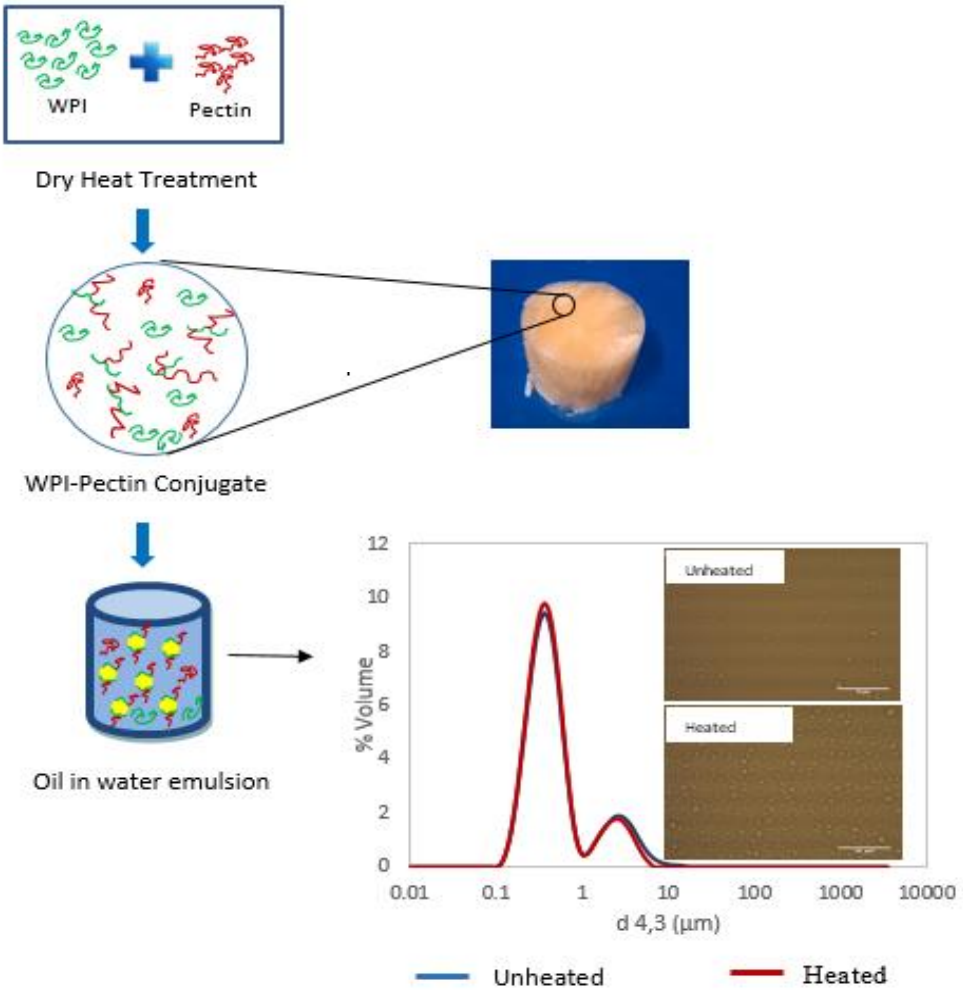
5.4 Conclusion

The results showed that NMR can be used as an alternative method to study the interaction between proteins and polysaccharides. The spectra obtained from the measurement contained highly important information such as the diffusion behaviour of the WPI and LMP. Using the extracted data, the quantification of the free and reacted WPI in the mixtures and conjugates was successfully performed. In the case of electrostatic interaction between WPI and LMP, stronger interaction between WPI and LMP was found at a pH close to the IEP of the WPI, while at relatively neutral pH there was limited interaction between WPI and LMP. For WPI and LMP conjugates obtained by dry heat treatment, the amount of reacted WPI or the degree of interaction between WPI and LMP increased by extending the duration of dry heat treatment. Nevertheless, it should be noted that a significant interaction between WPI and LMP took place during the first two days of incubation time. Besides extending the duration of the dry heat treatment, a higher degree of interaction was also obtained by increasing the concentration of LMP in the conjugates. NMR has been successfully used to monitor the

formation of WPI-LMP conjugates as a function of incubation time which was difficult to be performed with QCM-D. Despite of showing different value with that obtained from chemical analysis (TNBS measurement), the results from both NMR and chemical analysis showed the same trend. The difference observed was caused by the different principle used in the measurement.

CHAPTER 6

THE INFLUENCE OF DEGREE OF ESTERIFICATION ON THE EMULSIFYING ACTIVITY OF WHEY PROTEIN-PECTIN CONJUGATES AND ON THE HEAT STABILITY OF THE EMULSIONS STABILIZED BY THESE CONJUGATES



Abstract

The influence of the degree of esterification (DE) on the performance of WPI-Pectin conjugates in stabilizing emulsions was evaluated. Prior to the conjugate preparation, the pectins (both High Methoxyl and Low Methoxyl Pectin) were dialyzed to remove simple sugars which can also be involved in the conjugate preparation and thus influence the results. WPI-Pectin conjugates were prepared by mixing the protein and polysaccharides at a ratio of 2:1. The mixtures were then dried and incubated at a temperature of 60^{o}C and relative humidity of 74% (dry heat treatment). The progress of the Maillard reaction during dry heat treatment was monitored by measuring the formation of brown pigment at 420 nm. The conjugates produced were then used to stabilize 10% O/W emulsions which were prepared at pH 6.5 and 5. The emulsifying activity and the stability of the emulsions against creaming and heat induced droplet flocculation were studied.

It was found that the dialysis was effective in removing sucrose and dextrose from LMP and HMP, respectively. There was a significant reduction of the brown pigment production upon dry heat treatment of WPI and HMP when the pectin was first dialyzed. The results obtained in this study indicated that, generally, the emulsifying activity of the conjugates in the presence of HMP and LMP was comparable. By removing dextrose from the HMP, a longer incubation time did not give rise to WPI-HMP conjugates with a poor emulsifying activity as reported previously. After 16 days of incubation, the WPI-HMP conjugates exhibited excellent emulsifying activity. No noticeable difference in the viscosity of the emulsions stabilized by WPI-HMP and WPI-LMP conjugates was found. The heat stability of the emulsions stabilized by WPI-pectin conjugates was not influenced by the DE either. The emulsions stabilized by the WPI-LMP and WPI-HMP conjugates were stable against heat induced droplet aggregation after heating at 80^{o}C for 20 minutes. Nevertheless, the creaming stability of the emulsions both before and after heating was influenced by the DE of pectin. Emulsions were more stable against creaming in the presence of pectin with a higher DE, despite the fact that the emulsions stabilized by WPI-LMP conjugates had a higher electrophoretic mobility. Hence, it was suggested that the stabilization mechanism of the conjugates was based more on steric stabilization than electrostatic repulsion. Nevertheless, the role of electrostatic stabilization could not be completely ruled out.

6.1 Introduction

Milk is a material rich in essential nutrients (Haug, Høstmark, & Harstad, 2007) which is highly prone to microbial growth. Accordingly, heat treatment is necessary in the milk industry to prolong the shelf life of milk (De Jong, 1997; Wijayanti, Bansal, Sharma, et al., 2014). The heat treatment of milk should be performed in a way that it can eliminate the microorganisms without destroying the nutrients present (De Jong, 1997). Protein stability against heat is still a big issue in the milk industry, for example in the production of concentrated milk. Upon processing of milk, globular proteins, such as whey proteins, undergo protein denaturation followed by aggregation due to their low heat stability. Heat induced protein denaturation followed by aggregation can lead to fouling in heat exchangers which can reduce their heat transfer efficiency (De Jong, 1997). During heat treatment of milk, fouling creates a major problem (Simmons, Jayaraman, & Fryer, 2007). Furthermore, the protein deposit on the walls of heat exchangers can induce microorganism growth. As a result, it can lead to an increase in cleaning costs which is undesirable (De Jong, 1997). Most importantly, protein denaturation and aggregation will alter the properties and characteristic of the final product. Therefore it is utmost important to tackle this problem.

Milk consists of different types of proteins such as caseins which represents 80% of the milk proteins and whey proteins (Haug, et al., 2007). Whey proteins are globular proteins known to be highly heat labile (Haug, et al., 2007). They consist of different fractions, namely β-lactoglobulin, α-lactalbumin, lactoferin, and Bovine Serum Albumin (BSA), with β-lactoglobulin being the major fraction (Haque, et al., 2013; Vasbinder, et al., 2003). Whey proteins, in particular β-lactoglobulin, play an important role in the milk fouling due to their heat lability (Georgiadis & Macchietto, 2000; Simmons, et al., 2007). While caseins are rather heat stable (De Kruif, 1999), interaction of caseins with denatured whey proteins can lead to protein aggregation between caseins and whey proteins. Upon heat treatment of milk, the unfolded whey proteins can aggregate with other unfolded whey proteins, or with other proteins such as caseins resulting in β-lactoglobulin coated casein micelles (Vasbinder, et al., 2003).

Since heat treatment is normally performed at temperatures above the denaturation temperature of the whey proteins, different methods to improve the heat stability of whey proteins have been proposed, such as enzymatic modification (Damodaran, 2005), blocking of

the sulfhydryl group (Wijayanti, Bansal, Sharma, et al., 2014), combining proteins with polysaccharides through electrostatic interaction (Ye, 2008) and covalent complexation via a Maillard type reaction (Einhorn-Stoll, et al., 2005; Kato, 2002). Covalent complexation of whey proteins and pectin has been reported to be effective in improving the emulsifying activity and heat stability of whey proteins (Drapala, et al., 2016a; Setiowati, et al., 2017; Setiowati, et al., 2016). Upon dry heat treatment, whey proteins are covalently linked to polysaccharides through a Maillard type reaction (Dickinson, 2008; Kato, 2002). This type of interaction has been reported to be able to withstand changes of pH and ionic strength better than an electrostatic interaction (Dickinson, 2008). There have been reports on the dry heat treatment of whey proteins with different types of monosaccharides (Aoki, et al., 1999; Bernal, et al., 1985b) and oligosaccharides (Bouhallab, Morgan, Henry, Mollé, & Léonil, 1999; Bu, et al., 2015). However, dry heat treatment in the presence of monosaccharides and oligosaccharides is prone to the advanced stage of the Maillard reaction and polymerization (Jiménez-Castaño, Villamiel, et al., 2005; Kato, 2002), which in turn can reduce the functionality of the whey proteins (Jiménez-Castaño, Villamiel, et al., 2005). This phenomenon can be well avoided when a polysaccharides is used. However, due to its lower reactivity, the utilization of long chain polysaccharides has a drawback: it might require a longer dry heat treatment than mono- and oligosaccharides in order to form conjugates with the desirable properties. Several researchers have proven that, despite the fact that they are less reactive than shorter chain saccharides, long chain polysaccharides offer a great functionality when they are conjugated with proteins (Akhtar, et al., 2007; Benichou, Aserin, Lutz, et al., 2007; Diftis, et al., 2006a; Zhu, et al., 2010).

This study focuses on the utilization of the long chain polysaccharide pectin to form protein-polysaccharide conjugates in order to improve the heat stability of whey proteins. Pectin was chosen due to its abundant availability in nature. It is a long chain and anionic polysaccharide consisting of α- D galacturonic acid (Lootens, et al., 2003). Pectin itself has been widely used in food application as a thickening agent, gelling agent (Lootens, et al., 2003), and stabilizer (Pereyra, Schmidt, & Wicker, 1997). In nature, some of the carboxyl groups in pectin are esterified with methanol (Levigne, Thomas, Ralet, Quemener, & Thibault, 2002) and depending on the amount of esterified carboxyl groups, pectins can be grouped into Low and high methoxyl pectin (LMP and HMP, resp.). Low methoxyl pectin is characterized by having a

degree of methylation below 50%, while high methoxyl pectin has a degree of methylation above 50% (Lootens, et al., 2003). The DE of pectin determines its functionality, especially its gelling properties.

Our previous study has indicated that 2 days of dry heat treatment of the mixture of low methoxyl pectin (LMP) and WPI was sufficient to produce WPI-LMP conjugates with superior functionality and heat stability (Setiowati, et al., 2017). A comparative study on the functionality of protein-polysaccharide complexes prepared with low and high methoxyl pectin was reported by Neirynck *et al.* (2004). However, this study might not reflect the true influence of the degree of esterification (DE) of the pectin used on the functionality of the conjugates since the LMP and HMP used in that study contained simple sugars which were added by the supplier to standardize the performance of the pectin (Chan, et al., 2016; May, 1990). Upon dry heat treatment for 16 days, the WPI-HMP conjugates produced were reported to have a poor functionality, which was suggested to be due to the advanced Maillard reaction between the sugar and whey proteins. With this regard, this study was designed to investigate the influence of the DE on the functionality of WPI-pectin conjugates by minimizing the influence of sugar present in the pectin. In addition, their influence on the heat stability of the emulsions stabilized by the conjugates was also evaluated.

In this study, the LMP and HMP were dialyzed prior to the conjugate formation to eliminate the simple sugars present in the pectin. The conjugates were then used to stabilize oil in water emulsions to assess the emulsifying activity of the conjugates. This was then followed by subjecting the emulsions to a heat treatment in order to test the heat stability of the emulsions.

6.2 Materials and Methods

6.2.1 Materials

WPI was purchased from Davisco Foods International Inc. (Le Sueur, MN, USA). Protein analysis revealed that the WPI contained approximately 97.7% protein, whereby 85% of the protein is β-lactoglobulin (Van der Meeren, et al., 2005). The WPI was reported by the manufacturer to contain a limited amount of lactose (0.5-1%). Therefore, the presence of lactose was expected to have no significant influence during dry heat treatment. Low

methoxyl pectin (LMP) with DE of 33-38% (Unipectin OB700) and high methoxyl pectin (HMP) (Unipectin AYD250) with DE of 69-74% were obtained from Cargill (Ghent, Belgium) and contained 89.6% and 90.5% of dry matter, respectively (Neirynck, et al., 2004). According to the manufacturer, 5% of sucrose and 40% of dextrose were added to the LMP and HMP, respectively. This was supported by the results of gas chromatography measurements which revealed that there was reducing sugar present in the HMP, while no reducing sugar was detected in the LMP (data not shown). Sunflower oil purchased from a local supermarket was used for emulsion preparation.

6.2.2 Methods

6.2.2.1 Dialysis

According to the manufacturer, HMP and LMP contained 40% of glucose (also indicated as dextrose) and 5% of sucrose, respectively. In this study, the pectins were purified by means of dialysis to remove the residual dextrose and sucrose from the pectin. 1% of HMP and LMP solution (w/v) was prepared in Milli-Q water and the pH of the solutions was adjusted to 7.0. NaN_3 was added into the solutions to prevent microbial growth during dialysis. 10 mL of the pectin solutions were then put into a dialysis tube with a molecular weight cut off of 12-14000 Da and a diameter of 15.9 mm (Medicell membranes, UK). The membranes were then put into a container filled with 150 mL of Milli-Q water. To monitor the sugar transport during dialysis, the total carbon (TC) content of the HMP and LMP solutions inside the dialysis membranes as well as of the Milli-Q water used to dialyze the pectins was measured using a TOC-5000 (Shimadzu). The measurement was performed after 0, 24, and 48 hours. To determine the TC content of the sample, a calibration curve consisting of TC contents of 0, 25, 50, and 100 ppm was constructed. For this purpose, pottasium hydrogen phthalate (Nacalai Teque, Japan) was used to prepare the standard solution. For simulation of an ideal condition in which all the sucrose and dextrose were transported to the Milli-Q water, the TC content of sucrose and dextrose solution containing 0.05% and 0.4%, respectively, was measured upon 16 fold dilution. This concentration was chosen to match the concentration present in the 1% of LMP and HMP solutions. For the conjugate preparation, the pectin solution was collected and lyophilized after dialysis. The lyophilized pectin was then rehydrated with demineralized water to its initial volume (before dialysis) to be used for conjugates

preparation. Alternatively, the dried pectin can also be stored at room temperature for further use.

6.2.2.2 Conjugates preparation and emulsion preparation

WPI-pectin mixtures and conjugates, prepared with dialyzed and non-dialyzed LMP and HMP, were prepared following the method described in section 2.2.2. The conjugates and mixtures were prepared at a WPI:pectin ratio of 2:1. In this study, the dry heat treatment was performed for 0, 1, 2, and 16 days.

10% of oil in water emulsions stabilized by 0.5% of WPI, 0.5% WPI-Pectin mixture, and 0.5% WPI-pectin conjugates were prepared at pH 6.5 and 5.0 (in the presence of 30 mM NaCl). The emulsions were prepared according to the method described in section 3.2.3

6.2.2.3 Heat coagulation test

The heat coagulation test of the emulsions was conducted based on the method developed by Kasinos, et al. (2015), as described in section 3.2.4.

6.2.2.4 Particle size analysis

The particle size distribution analysis was performed using a Mastersizer 3000 (Malvern Instruments Ltd, Malvern, UK) as explained in section 3.2.5.

6.2.2.5 Microscopy observation

The oil droplets within the emulsions were observed using a CX40 light microscope (Olympus GmbH, Hamburg, Germany) equipped with an Axiocam ERc5s camera (ZEISS, Germany). The observation was done at a magnification of 100X with addition of immersion oil to improve the image quality obtained from the microscope.

6.2.2.6 Viscosity.

An LV-DVII+pro portable viscometer (Brookfield) was employed to measure the viscosity of the emulsions as described in section 3.2.7. Spindle SC-18 was used to measure emulsions with low viscosity, while spindle SC 34 was used to measure samples with a gel like structure.

6.2.2.7 Electrophoretic mobility

The electrophoretic mobility (EM) of the unheated emulsions was measured using a Zetasizer 2c (Malvern Ltd, UK) at pH 5.0 and 6.5 following the method described in section 3.2.9.

6.2.2.8 Accelerated creaming stability evaluation

The creaming stability of the emulsions was evaluated using a LUMiFuge 116 (LUM GmbH, Germany). The measurements and data analyses were performed based on the method described in section 3.2.8.

6.2.2.9 Statistical analysis

The statistical analysis was generated using SPSS 22 (IBM). Two-way Anova was performed on the results of droplet size and creaming velocity measurement at a significance level of 5% to find the influence of DE on the emulsions stabilized by WPI in the presence of non-dialyzed pectin. The same test was performed for emulsions stabilized by WPI in the presence of dialyzed pectin. The influence of heat treatment and incubation time on the droplet size and creaming velocity of the emulsions was also tested using Two-way Anova at a significance level of 5%. A Paired *t*-test at a significance level of 5% was performed to test the influence of dialysis on the particle size and creaming velocity of the emulsions. Univariate ANOVA was performed on the results of electrophoretic mobility measurements at a significance level of 5%.

6.3 Results and Discussion

The influence of the degree of esterification on the heat stability of WPI-pectin conjugates has been previously studied by Neirynck, et al. (2004). These authors reported that conjugation of WPI with LMP resulted in conjugates with improved emulsifying activity. In contrast, the conjugates prepared with HMP had a poor solubility and emulsifying activity which gave rise to emulsions with big droplet sizes. This phenomenon was attributed to the presence of sugar, i.e. dextrose in HMP. Since dextrose is a reducing sugar, it might interfere with the Maillard reaction between WPI and HMP. Therefore, the results obtained from this study might not truly represent the effect of DE. The present study was performed using LMP and HMP with

the same specifications. According to the manufacturer, the LMP and HMP used in our study contained 5% of sucrose and 40% of dextrose, respectively. In order to eliminate the influence of dextrose and sucrose, the HMP and LMP samples were dialyzed prior to the conjugate formation.

6.3.1 Removal of sugar with dialysis

Dialysis was performed to remove the added sugar from the HMP and LMP powder. As the HMP and LMP solutions were dialyzed against Milli-Q water, the total carbon (TC) content of the Milli-Q water outside the dialysis membrane was determined to monitor the sugar transport from the inside of the membrane to the Milli-Q water. Figure 6.1 shows the TC content of the Milli-Q water and of the pectin solution inside the dialysis membrane as a function of time.

Table 6.1. Total carbon content (ppm) of the LMP and HMP solutions as well as of the Milli-Q water used to dialyze the pectin solutions as a function of dialysis time; the dilution factor of the internal phase was 100x, whereas the external phase was diluted only 10x.

	0 h		24 h		48 h	
	Internal (pectin)	External (Milli-Q)	Internal (pectin)	External (Milli-Q)	Internal (pectin)	External (Milli-Q)
LMP	36.02	0	25.48	1.07	24.22	1.83
HMP	42.14	0	15.6	10.48	13.76	12.38

It can be noticed that the TC content of the pectin solution and of the Milli-Q water presented in Table 6.1 was not balanced. This can be explained by the water transport phenomenon that took place: during dialysis, water was transported from the external phase (Milli-Q water) to the internal phase (pectin solution). Thus, dilution took place in the internal part of the membranes. Using the mass balance equation below, it was possible to calculate the amount of water transported to the pectin solution.

$$V_{internal} = \frac{(V_{pectin} \times TC_{Pectin} \times DF_{Pectin}) - (V_{External} \times TC_{External} \times DF_{External})}{(TC_{Internal} \times DF_{Internal})} \quad \text{(Equation 6.1)}$$

Since:

$$V_{External} + V_{Internal} = 160 \text{ mL} \quad \text{(Equation 6.2)}$$

Thus:

$$V_{External} = 160 - V_{Internal} \quad \text{(Equation 6.3)}$$

$$V_{internal} = \frac{(V_{pectin} \times TC_{Pectin} \times DF_{Pectin}) - (160 \times TC_{External} \times DF_{External})}{(TC_{Internal} \times DF_{Internal}) - (TC_{External} \times DF_{External})} \quad \text{(Equation 6.4)}$$

V_{pectin} is the volume of the HMP or LMP solution brought into the dialysis membrane (i.e. 10 mL), TC_{Pectin} is the original total carbon content of the HMP or LMP solution (42.14 and 36.02 ppm, respectively). $V_{external}$ is the volume of the Milli-Q water outside the dialysis membrane after dialysis, while $V_{internal}$ is the final volume of HMP or LMP solution inside the dialysis membrane after dialysis. $TC_{external}$ and $TC_{internal}$ are the Total Carbon content of the Milli-Q water and of the HMP/LMP solutions inside the dialysis membrane after dialysis, respectively. Prior to the dialysis, 10 mL of HMP or LMP solution was put into the dialysis membrane and 150 mL of Milli-Q water was used to dialyze the solutions. Thus, the sum of $V_{external}$ and $V_{internal}$ is 160 mL. The dilution factor (DF) for the TC_{pectin} and $TC_{internal}$ was 100, while for the $TC_{external}$ it was 10.

In Equation 6.1, the only unknown factors are $V_{external}$ and $V_{internal}$, whereby the value of TC_{pectin}, $TC_{internal}$, and $TC_{external}$ can be found in Table 6.1. Since the sum of $V_{external}$ and $V_{internal}$ is known (Equation 6.2), $V_{external}$ in Equation 6.1 can be substituted with Equation 6.3 which leads to Equation 6.4. The results of the calculation are presented in Table 6.2.

Table 6.2. Volume (mL) of the pectin solution inside the dialysis membrane (internal) and of the Milli-Q water (external) after 24 and 48 hours of dialysis

Dialysis time (h)	LMP		HMP	
	Internal	External	Internal	External
0	10.0	150.0	10.0	150.0
24	13.5	146.5	17.5	142.65
48	13.8	146.2	17.8	142.2

After 24 hours, it was found that approximately 3.5 mL and 7.5 mL of water was transported from the Milli-Q compartment to the LMP and HMP solution, respectively, which is driven by the concentration difference between the internal and external compartment of the dialysis membrane. Extension of the dialysis to 48 hours did not further induce a remarkable water transport: it was found that after 48 hours the additional amount of water transported was less than 0.5 mL. After 48 hours, the total volume of water transported through the membrane was 3.8 and 7.8 mL, respectively. Since the final volume of the pectin solution increased due to water transport, the $TC_{internal}$ presented in Table 6.1 should be recalculated to have the dilution-corrected value, i.e. the value that would be obtained at a fixed volume of 10 mL:

$$TC_{corrected} = V_{Internal}\, x\, TC_{Internal}/V_{pectin} \quad \text{(Equation 6.5)}$$

These dilution-corrected values can be found in the right part of Figure 6.1, which shows the reduction of the TC content of the pectin solution as a function of dialysis time. From the left part of Fig. 6.1, the rate of sugar transport from the pectin solutions to the Milli-Q water during dialysis can be seen: the TC content of the Milli-Q water increased as sugar was transported from the pectin solution to the outer part of the membrane.

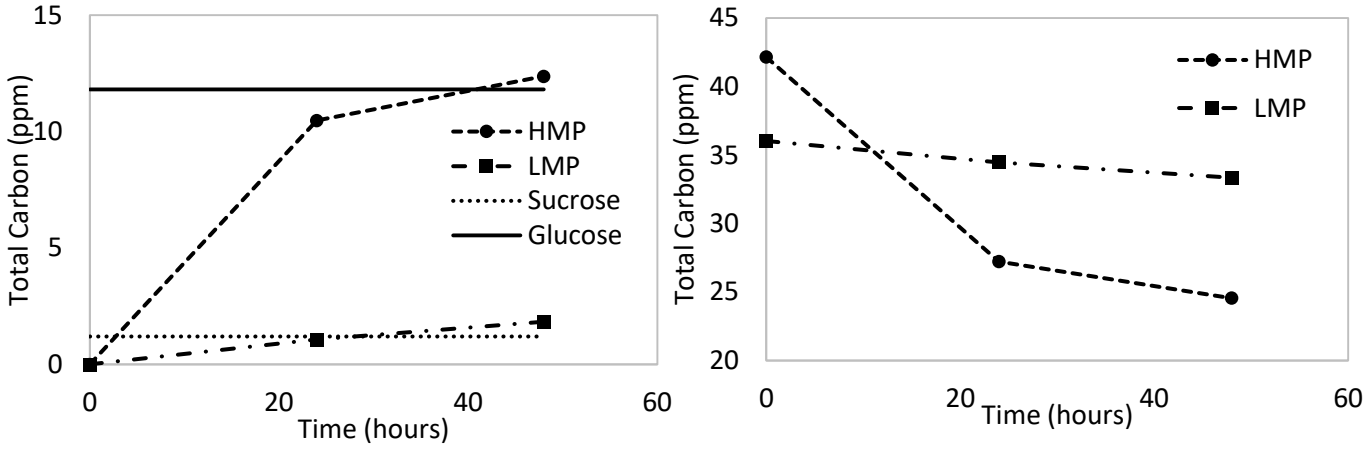


Figure 6.1 Dilution-corrected Total Carbon content (ppm) of the Milli-Q water (left) and of the HMP and LMP solutions (right) as a function of dialysis time as well as of the sucrose and dextrose solutions, as a reference.

In order to estimate the expected TC in the external phase when an equilibrium condition is reached, the TC content of a sample obtained by mixing either 10 mL of 0.05% of sucrose (corresponding to the sucrose content in 1% LMP) or 10 mL of 0.4% dextrose (corresponding

to the dextrose content in 1% HMP) with 150 mL of Milli-Q water was determined upon 10 times dilution. The TC contents of these simulated external liquid compositions were 1.19 and 11.82 ppm for sucrose and dextrose, respectively.

It can be seen in Figure 6.1 that after 48 hours, the $TC_{external}$ of LMP and HMP was close to the expected values ($TC_{external,expected}$) obtained from the sugar solutions, i.e. 1.19 ppm for Sucrose (LMP) and 11.82 ppm for dextrose (HMP). Based on the Total Carbon data, the effectiveness of the dialysis was calculated using equation 6.6.

$$Sugar\ removal\ efficiency: \frac{TC_{external}}{TC_{external,expected}} \times 100\% \qquad \text{(Equation 6.6)}$$

It was found that after 24 hours, 89% and 88% of the sugar was removed from the LMP and HMP solutions, respectively. Dialysis for 48 hours was found to be able to remove all sucrose and dextrose from the LMP and HMP solutions, respectively. In fact, based on Equation 6.6, the calculated removal efficiency was 153% and 114% for LMP and HMP after 48 hours of dialysis, respectively. This overestimation could be due to a limited permeation of low molecular weight species of pectin. Based on these results, the HMP and LMP solutions for the conjugate preparation were dialyzed for 48 hours.

The removal of simple sugars from the HMP and LMP powder is important since the presence of these sugars, and especially dextrose, may interfere during the formation of conjugates. Sucrose is a disaccharide composed of glucose and fructose linked to each other via a glycosidic bond and is included in the non-reducing sugar group. Dextrose, on the other hand, is a monosaccharide and a reducing sugar. Due to this, unlike sucrose, dextrose will be actively involved in the Maillard reaction. When it is not removed, upon incubation of HMP with WPI, the carbonyl groups of dextrose will compete with the carbonyl groups of HMP to interact with the amino groups of WPI. Therefore, the conjugates produced might contain not only WPI-HMP conjugates but also WPI-dextrose conjugates. It has been known that interaction between long chain polysaccharides with proteins via Maillard reaction takes hours to several days depending on the composition and the dry heat treatment conditions (Einhorn-Stoll, et al., 2005; Kato, et al., 1990; Schmidt, et al., 2016). If the sucrose in LMP undergoes hydrolysis resulting in fructose and glucose, they will also be able to participate in the Maillard reaction during dry heat treatment of LMP and WPI which, in this study, is undesirable. In this study,

the WPI was not dialyzed since it contained only a very limited amount of lactose which was expected to have a negligible influence on the formation of the conjugates.

6.3.2 Formation of brown colour and intermediate products

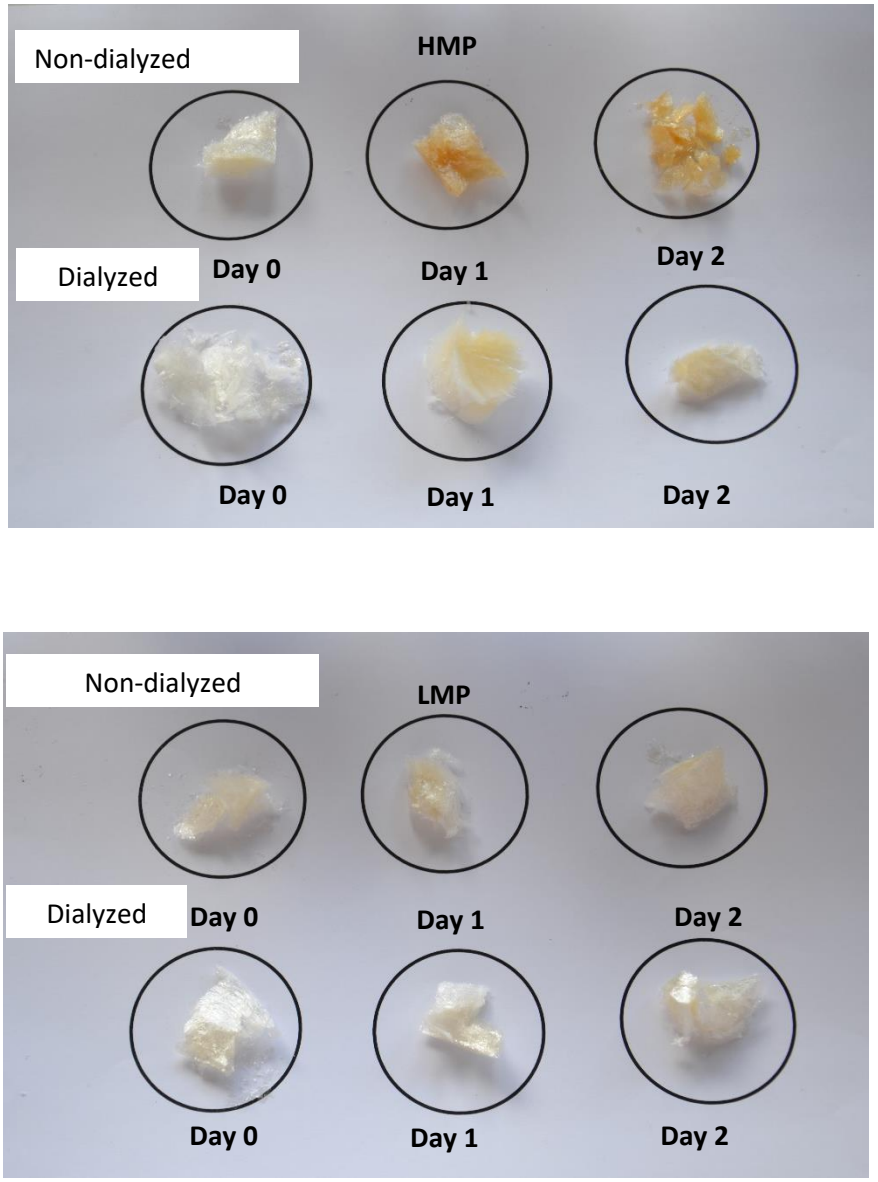


Figure 6.2. Colour difference between mixtures of WPI and pectin (i.e. Day 0) and their respective conjugates (obtained after 1 or 2 days of dry heat treatment) prepared with dialyzed and non-dialyzed HMP (Top) and LMP (Bottom) pectin, respectively.

Figure 6.2 shows the WPI-pectin conjugates obtained after 0, 1, and 2 days of dry heat treatment. Based on the image, it can be seen that before dialysis, the conjugates prepared

with HMP had an intense yellow to brown colour. The intensity of the brown colour decreased considerably when the conjugates were prepared with the dialyzed HMP. As for conjugates prepared with LMP, there was no remarkable difference in the colour of the conjugates prepared with dialyzed and non-dialyzed LMP. The Maillard reaction is a complicated reaction which consists of three different stages namely the early, intermediate/advanced, and final stage. Some of the products from this type of reaction are brown pigments. The formation of brown pigments is especially very intensive at the final stage of the Maillard reaction (Aoki, et al., 1999). The formation of this brown colour can be followed by measuring the absorbance of an aqueous solution of the sample at 420 nm. Furthermore, formation of the intermediate products of the Maillard reaction can also be followed by measuring the absorbance of an aqueous solution of the sample at 294 nm. Figure 6.3 shows the formation of brown colour (left) and intermediate products (right) as a result of the Maillard reaction as a function of incubation or dry heat treatment time.

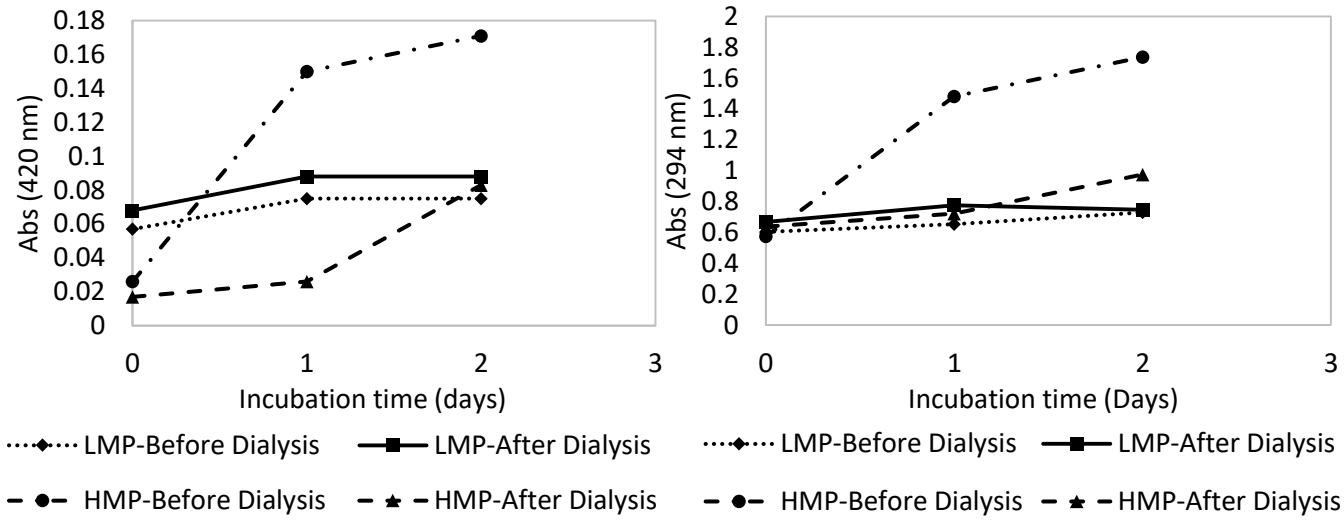


Figure 6.3 Brown colour (left) and intermediate products (right) formation during dry heat treatment of WPI-LMP and WPI-HMP as a function of incubation time, as evaluated from absorbance measurements of a 2 mg/mL solution in distilled water at 420 and 294 nm, respectively.

It can be noticed in Fig. 6.3 that there was no remarkable difference in the absorbance of LMP-WPI conjugates at both 420 and 294 nm produced with dialyzed and non-dialyzed LMP: the conjugates produced with both dialyzed and non-dialyzed LMP exhibited the same trend. This was expected since sucrose is a non-reducing sugar. Hence, removal of sucrose from LMP did

not have a considerable effect on the Maillard reaction between WPI and LMP. On the other hand, a noticeable difference between HMP-WPI conjugates prepared with dialyzed and non-dialyzed HMP can be observed in Fig. 6.3.

Upon dry heat treatment of WPI and non-dialyzed HMP, a significant amount of brown colour was produced during the first day of dry heat treatment, which levelled off during the second day of dry heat treatment. Nevertheless, the intensity of brown colour formed upon incubation of WPI and dialyzed HMP was relatively low. This phenomenon was believed due to the removal of dextrose from the HMP. Dextrose is a reducing sugar and is more reactive than pectin. Therefore the high intensity of brown colour produced in the first day of dry heat treatment was believed to be due to Maillard reaction between dextrose and WPI. After the removal of dextrose from HMP, it can be observed that the formation of brown colour was limited during the first day of dry heat treatment and increased on the second day. This further proved that the high rate of brown colour formation on the first day of dry heat treatment was caused by the presence of dextrose. Comparing the HMP and LMP, it can be seen that conjugation of WPI and HMP produced more brown pigment as compared to WPI and LMP. This could mean that the Maillard reaction in WPI-HMP progressed faster than that in WPI-LMP.

During Maillard reaction, intermediate products are formed and can be transformed into brown pigments as the reaction progresses (Ajandouz, Tchiakpe, Ore, Benajiba, & Puigserver, 2001). As for the formation of intermediate products, the trend exhibited was comparable to that of brown colour formation, as was expected (Lertittikul, et al., 2007). The production of intermediate products in WPI-LMP conjugates was less influenced by dialysis. On the other hand, the formation of intermediate products in the presence of dialyzed HMP was much lower than that in the presence of non-dialyzed HMP. Similar to the brown pigment production, the dry heat treatment of WPI-HMP mixtures also produced a higher amount of intermediate products as compared to WPI-LMP mixtures. The results presented in Figure 6.3 also indicate that the formation of intermediate products and brown pigment has not reached a maximum. Since sugar removal decreased the formation of brown pigment and intermediate products significantly, the results suggested that the sugar, especially dextrose, participated in the Maillard reaction during dry heat treatment.

6.3.3 Functionality of the conjugates

6.3.3.1 Emulsifying activity and heat stability

The emulsifying activity of the conjugates was evaluated in 10% oil in water (O/W) emulsion. The emulsifying activity of the conjugates containing HMP was compared to that of the conjugates containing LMP. Furthermore, the influence of DE on the heat stability of the emulsions was also evaluated.

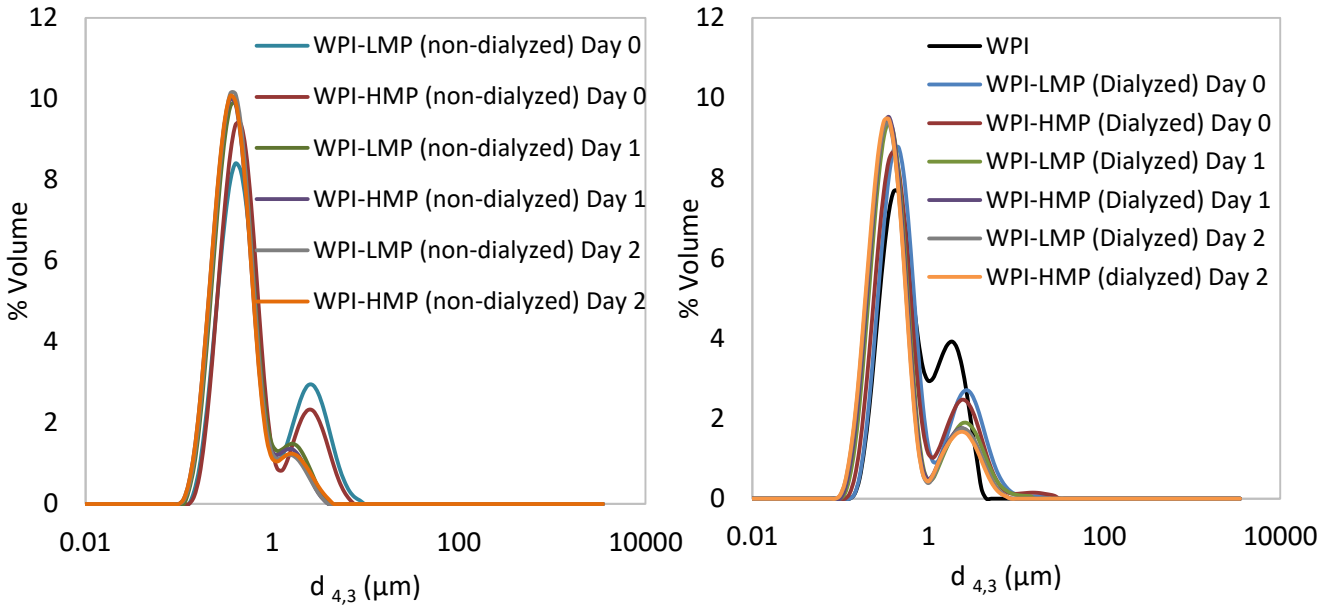


Figure 6.4 Volume-weighed particle size distribution of unheated 10% o/w emulsions stabilized by 0.5% of WPI or WPI-pectin conjugates prepared with dialyzed (right) and non-dialyzed (left) pectin.

The volume-weighted average droplet size of the emulsions stabilized by WPI and WPI-pectin conjugates is presented in Table 6.3. The results indicate that conjugation of WPI with pectin improved the emulsifying activity of WPI. WPI-pectin conjugates stabilized emulsions had a smaller droplet size than those stabilized by WPI only and WPI-pectin mixtures. Statistical analysis showed that there was a slight influence of DE on the particle size of the emulsions stabilized by WPI in the presence of non-dialyzed pectin ($p<0.05$); in the presence of HMP, the emulsions had a slightly smaller size. However, the influence of DE was not observed when the pectin was dialyzed ($p>0.05$). In addition, it can be noticed that the emulsions stabilized by WPI-pectin conjugates prepared with dialyzed pectin had a bigger droplet size than those

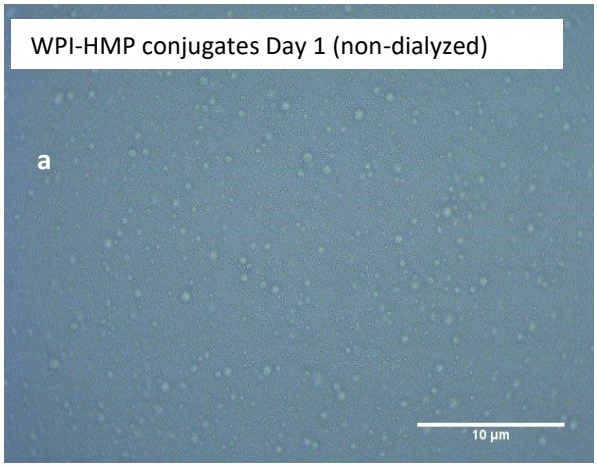
prepared with non-dialyzed pectin ($p<0.05$) which was also supported by the microscopic images of the emulsions (Fig. 6.5 a, b, g, i).

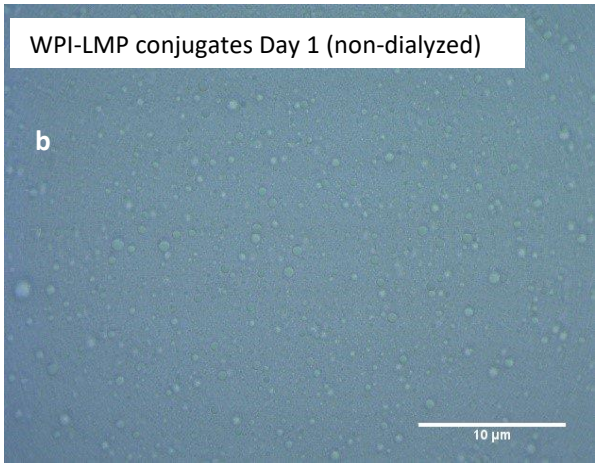
Table 6.3 Volume-weighed average diameter ($d_{4,3}$, in μm) of 10% o/w emulsions stabilized by 0.5% of WPI, WPI-LMP mixture, WPI-HMP mixture, WPI-LMP conjugates, or WPI-HMP conjugates

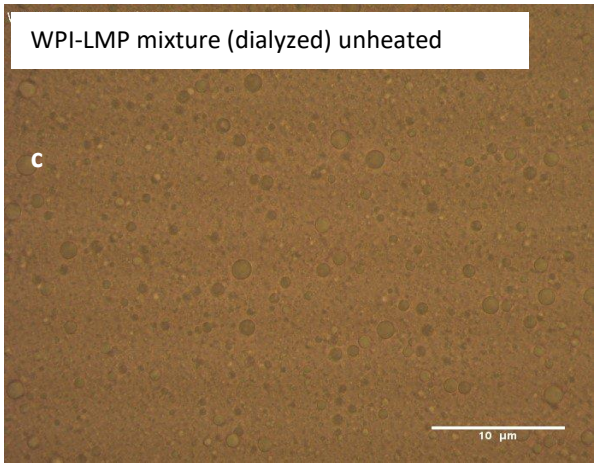
Sample	Incubation time (day)	Non-dialyzed		Dialyzed	
		Unheated	Heated	Unheated	Heated
WPI		0.90±0.03	9.09±0.18		
WPI-LMP	0	$1.16±0.01^{aA}$	$11.47±1.03^{B}$	$1.29±0.03^{aA}$	$20.17±0.83^{B}$
	1	$0.60±0.00^{bA}$	$0.62±0.00^{A}$	$0.75±0.01^{bA}$	$0.76±0.01^{b}$
	2	$0.56±0.00^{cA}$	$0.61±0.00^{A}$	$0.77±0.01^{bA}$	$0.65±0.00^{A}$
WPI-HMP	0	$0.94±0.00^{aA}$	$5.18±0.40^{B}$	$1.24±0.01^{aA}$	$10.25±0.52^{B}$
	1	$0.56±0.00^{bA}$	$0.64±0.00^{A}$	$0.79±0.00^{bA}$	$0.76±0.00^{b}$
	2	$0.57±0.00^{cA}$	$0.62±0.00^{A}$	$0.75±0.00^{cA}$	$0.77±0.00^{b}$

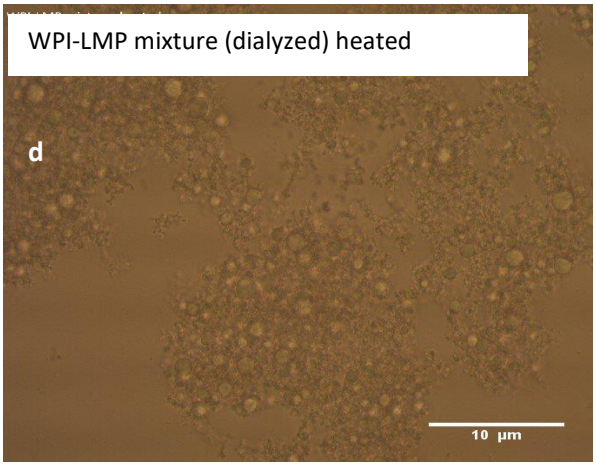
^{a,b} Means in the same column (with the same pectin source) followed by different lowercase letters are significantly different ($p<0.05$) (excluding data of the emulsions stabilized by WPI).
^{A,B} Means in the same row and group (non-dialyzed and dialyzed) followed by different uppercase letters are significantly different ($p<0.05$)(excluding data of the emulsions stabilized by WPI).

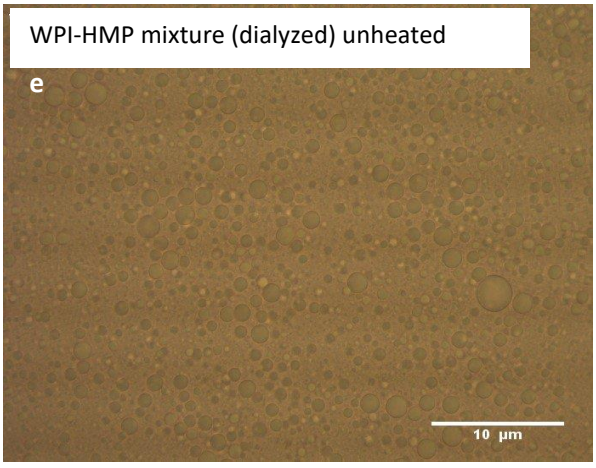
Based on Table 6.3 and Fig. 6.5 (c, d, e, and f), extensive heat induced droplet flocculation took place in emulsions stabilized by WPI and mixtures of WPI-pectin prepared using dialyzed and non-dialyzed pectin. Whereas emulsions stabilized by the WPI only and by mixtures of protein and polysaccharides showed a poor heat stability, emulsions stabilized by the conjugates prepared with HMP and LMP, both dialyzed and non-dialyzed, showed an excellent heat stability. Without dry heat treatment, the emulsions underwent a significant increase in their droplet size after heating. In contrast, when the emulsions were stabilized by WPI-pectin (dialyzed and non-dialyzed) conjugates (dry heat treated for 1 and 2 days), the droplet size of the emulsions was comparable before and after heating ($p<0.05$) (Table 6.3). With regard to the degree of esterification, there was no remarkable difference between the emulsifying activity of conjugates prepared with HMP and LMP and between the heat stability of the emulsions.

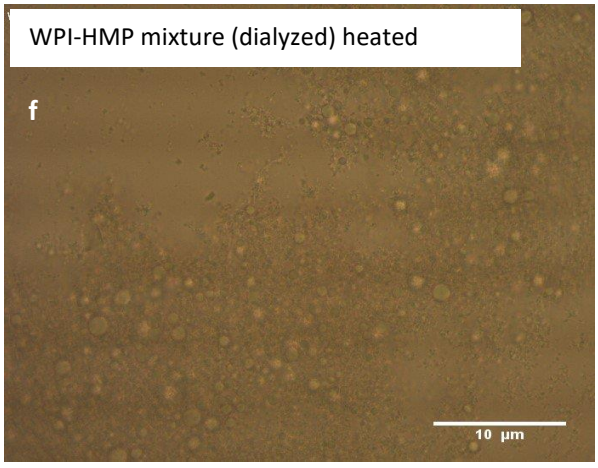

WPI-HMP conjugates Day 1 (non-dialyzed)
a
10 µm


WPI-LMP conjugates Day 1 (non-dialyzed)
b
10 µm


WPI-LMP mixture (dialyzed) unheated
c
10 µm


WPI-LMP mixture (dialyzed) heated
d
10 µm


WPI-HMP mixture (dialyzed) unheated
e
10 µm


WPI-HMP mixture (dialyzed) heated
f
10 µm

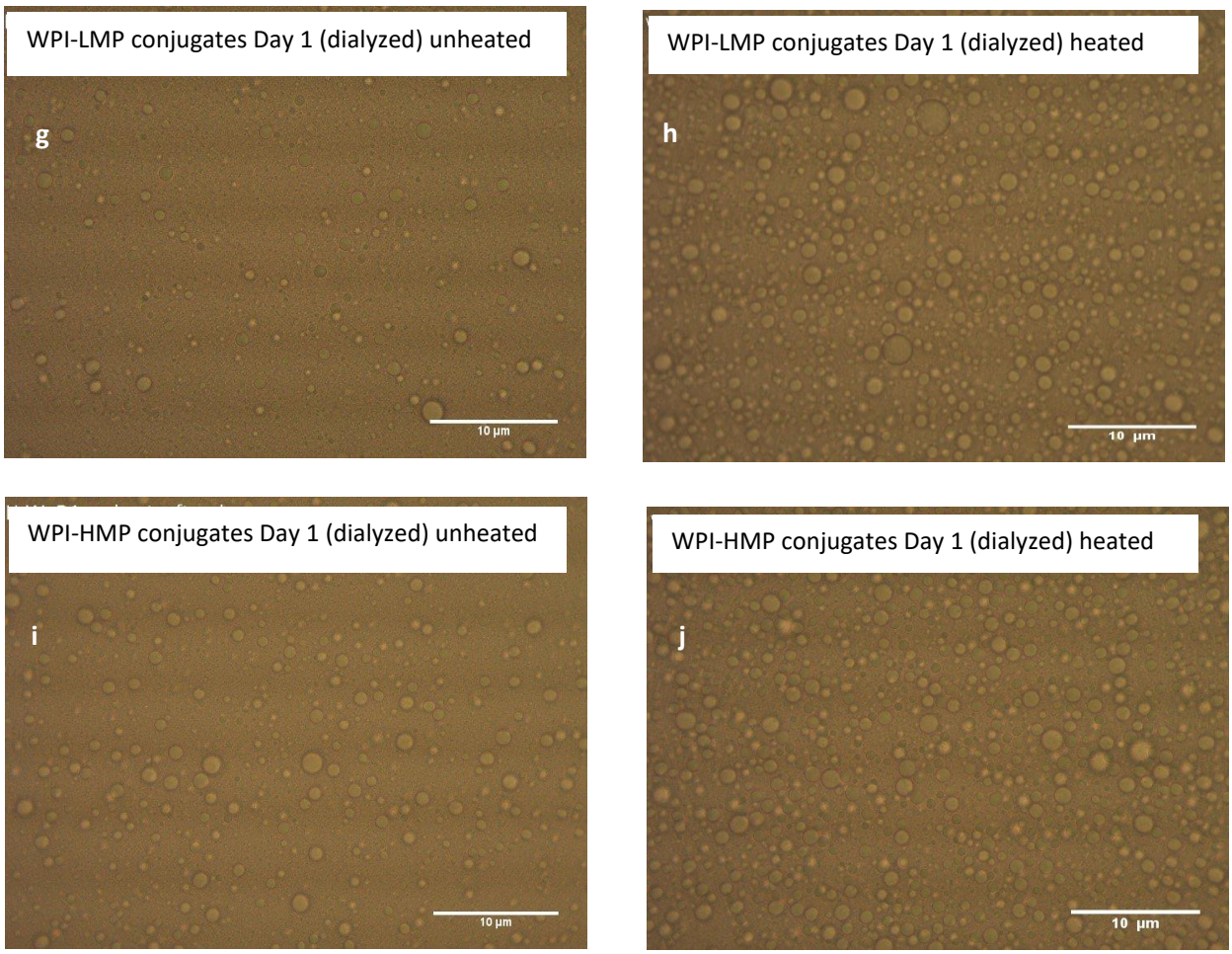


Figure 6.5. Microscopic images of unheated and heated emulsions stabilized by WPI-pectin mixtures and conjugates prepared with dialyzed and non-dialyzed LMP and HMP.

The results showed that the emulsions stabilized by WPI in the presence of dialyzed pectin produced a relatively bigger droplet size than in the presence of non-dialyzed pectin, which was also observed in the microscopic images of the emulsions ($p<0.05$). According to McClements (2002), the presence of electrolytes and sugar may influence the functionality of globular proteins (McClements, 2002). It was reported that the presence of low molecular weight sugar (e.g. sucrose and glucose) at low concentration reduces the surface activity of globular proteins, while at high concentration it improves their surface activity (Antipova & Semenova, 1997; McClements, 2002). However, the results in this study showed that the surface activity of WPI was higher in the presence of a low amount of sucrose and dextrose. Since the influence of dissolved species on globular proteins is highly related to the conformation of the proteins (McClements, 2002), it might be that the conjugates reacts

differently from the native globular proteins to the presence of a solute in the emulsions. However, further research needs to be done to prove this hypothesis.

Nevertheless, as the amount of sucrose and dextrose in the emulsions was very low, it was less likely that the sugars had impact on the emulsifying activity of WPI. The concentration of pectin in the emulsions was only 0.17%, meaning that there was only 0.07% of dextrose and 0.08% of sucrose in the emulsions stabilized by WPI-HMP and WPI-LMP, respectively.

Another possible explanation is that there was bridging flocculation induced by the higher MW fraction of the pectin. The molecular weight distribution of pectin is very broad. It has been mentioned in section 6.3.1 that the dialysis might also remove some of the lower MW fraction of the pectin. The presence of a higher MW fraction of pectin might induce bridging flocculation during emulsification which causes the emulsions to have bigger droplet size as supported by Figure 6.4 and 6.5. As it can be seen in Figure 6.4, the particle size distributions of all the emulsions were bimodal with the first and second peak representing small (submicron) and big (supermicron) droplets, respectively. Furthermore it can be observed that the intensity of the second peak increased as the emulsions were prepared in the presence of dialyzed pectin. It was obvious that the increase on the average droplet size of the emulsions was caused by the increase of the volume of the second peak which represents big droplets. Furthermore, there was almost no shift on the position of the first peak.

Neirynck, et al. (2004) reported that WPI-HMP conjugates which were dry heat treated for 14 days had a low solubility and the emulsions stabilized by the conjugates exhibited a poor stability against creaming at pH 4.5 and 5.5. For comparison, the emulsifying activity of WPI-HMP conjugates prepared with the dialyzed HMP at a pH close to the IEP of the proteins was evaluated. Contrary to the findings of Neirynck, et al. (2004), WPI-HMP conjugates prepared with the dialyzed HMP exhibited an excellent emulsifying activity at pH 5.0 even when they were incubated for a longer time (16 days) (Table 6.4). The conjugates were completely dissolved in the buffer and could produce emulsions with small droplet size (0.56 µm). Furthermore, the emulsion was stable against heat. The different behaviour is most likely due to the fact that advanced Maillard reaction took place between the dextrose present in the non-dialyzed HMP and WPI over the long dry heat treatment period used by Neirynck et al. (2004). Extensive glycation decreases the solubility of proteins due to the cross-linking and

polymerization which occurs at the advanced and final stages of the Maillard reaction (Oliver, et al., 2006). This reaction occurs partly due to the presence of sugar-derived dicarbonyl compounds which can attach to two lysine residues via their bifunctional groups leading to cross-linking of protein molecules (Oliver, et al., 2006). It has been reported previously that advanced Maillard reaction may produce protein-polysaccharide conjugates with low solubility, and thus poor stability of the emulsions stabilized by the conjugates (Jiménez-Castaño, Villamiel, et al., 2005). This can be observed in the WPI-HMP conjugates prepared with non-dialyzed HMP which was dry heat treated for 16 days (Fig. 6.6). In our study, this phenomenon was avoided since the dextrose was removed from HMP via dialysis. Moreover, this detrimental effect was also prevented by performing the incubation during a shorter time (i.e. 1 day) to limit the extent of the Maillard reaction (Table 6.4).

Table 6.4 Volume-weighted average droplet diameter ($d_{4,3}$) of 10% o/w emulsions stabilized by 0.5% WPI-HMP conjugates prepared at pH 5.0 in the presence of 30 mM NaCl.

Sample	Incubation time (day)	Before dialysis		After dialysis	
		unheated	heated	unheated	heated
WPI-HMP	0	0.77 ± 0.00^{aA}	9.39 ± 1.54^{aB}	0.72 ± 0.00^{aA}	0.72 ± 0.01^{aA}
	1	0.67 ± 0.00^{bA}	0.68 ± 0.00^{aA}	0.67 ± 0.00^{bA}	0.62 ± 0.00^{bB}
	16	not dissolved	not dissolved	0.56 ± 0.00^{cA}	0.59 ± 0.00^{cB}

[a,b] Means in the same column followed by different lowercase letters are significantly different ($p<0.05$).

[A,B] Means in the same row and group followed by different uppercase letters are significantly different ($p<0.05$).

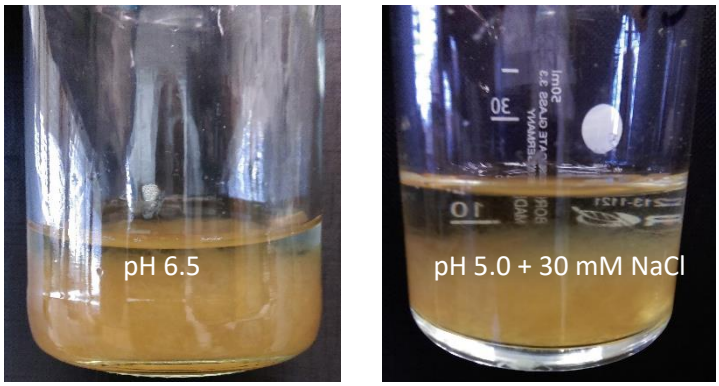


Figure 6.6. Insoluble WPI-HMP conjugates which were dry heated for 16 days, prepared using non-dialyzed pectin.

Interestingly, it was also found that without dry heat treatment (conjugation), the emulsions stabilized by a mixture of WPI and dialyzed HMP was stable against heat, while the same result was not obtained when the emulsions were stabilized by a mixture containing non-dialyzed HMP. A similar trend was also obtained for WPI-LMP mixtures (data are not shown). Previously, it was reported that upon heating emulsions stabilized by a mixture of WPI-LMP at 80^{o}C at pH 5.0 and in the presence of NaCl, the droplet size of the emulsions increased due to droplet flocculation (Setiowati, et al., 2017). However, in that study, the LMP was not dialyzed. Two possible explanations were proposed. First, the presence of simple sugar might influence the heat stability of whey proteins. Simple sugars were reported to increase the denaturation temperature of proteins by protecting the conformation of the native protein via steric exclusion (McClements, 2002). However once the proteins are heated at temperatures above their denaturation temperature, the presence of sugar will strongly enhance protein-protein interactions between the unfolded proteins resulting in a stronger gel and heat induced-droplet flocculation (Baier & McClements, 2005; Kulmyrzaev, Bryant, & McClements, 2000). The second possible explanation is the absence of free ions naturally present in pectin after dialysis. The LMP and HMP used in this study have been reported to naturally contain some cations such as Na^{+}, Mg^{2+}, Ca^{2+}, K^{+} (Neirynck, et al., 2004). The same authors reported that the LMP used contained 0.10%, 3.37%, 0.08%, and 0.01% (w/w) of K^{+}, Na^{+}, Ca^{2+}, and Mg^{2+}, respectively. On the other hand, the HMP was found to contain, 0.04%, 0.24%, 0.22%, and 0.01% (w/w) of K^{+}, Na^{+}, Ca^{2+}, and Mg^{2+}, respectively. The removal of these free ions was suggested to influence the interaction of WPI and pectin at pH 5.0 and their heat stabilizing activity as these ions can act as a counterions. This result, however, was not observed at pH 6.5. The emulsions stabilized by mixtures of WPI and pectin underwent droplet aggregation in the presence and absence of sugar (dialyzed and non-dialyzed pectin). It could be due to the fact that at pH 6.5, it was less likely that the WPI interacted with pectin in the mixture and thus was absent on the surface of oil droplets. Therefore, the WPI was prone to heat induced protein aggregation. Contrary to the phenomena observed in emulsions stabilized by mixtures of WPI and pectin, the emulsions stabilized by WPI-pectin conjugates exhibited an excellent heat stability in the presence and absence of sugar. Hence, it can be said that the heat stability of emulsions stabilized by WPI-pectin conjugates was solely due to the presence of the conjugates.

At short incubation times (up to 2 days), no influence of DE on the emulsifying activity of the conjugates or on the heat stability of the emulsions was observed. Nevertheless, in the case of non-dialyzed HMP, it was possible that the stabilizing effect was obtained from the combined effect of WPI-dextrose conjugates and WPI-HMP conjugates since an improvement of the heat stability of WPI due to conjugation with dextrose at 60^{o}C and for a short incubation time has been reported previously by Liu, Kong, Han, Sun, and Li (2014). For the non-dialyzed LMP, the presence of sucrose seemed not to influence the heat stability of the emulsions stabilized by WPI-LMP conjugates, since it is less likely that sucrose participates in Maillard type reactions. A previous study has reported that the presence of a low amount of sucrose was not able to stabilize β-lactoglobulin stabilized emulsions againts droplet flocculation upon heating at temperatures above the denaturation temperature of β-lactoglobulin (Kim, Decker, & McClements, 2003). However, these authors also stated that at a relatively high concentration (40%), sucrose was able to prevent heat induced droplet focculation. Therefore, to evaluate the influence of DE on the performance of the conjugates, it was important to eliminate the contribution of sugar. Hereby, based on the particle size measurements of the emulsions stabilized by the conjugates prepared with the dialyzed pectins, it was found that the DE did not have a remarkable influence on the emulsifying activity of the conjugates and on the heat stability of the emulsions.

The WPI-pectin conjugates were formed through interaction between WPI and pectin via Maillard type reactions which involve the amino groups of WPI and free carbonyl groups of pectin. Thus, it was expected that the degree of esterification would not influence the conjugate formation. Pectin is a long chain polysaccharide which has a linear and stiff structure. The degree of esterification in pectin influences the hydrodynamic and physical properties of pectin, such as the stiffness of the pectin structure (Harding, et al., 2017; Morris, Foster, & Harding, 2000; Tolstoguzov, 2000). Pectin with a lower degree of esterification has a more stiff structure (Morris, et al., 2000). Furthermore, pectin with a higher DE is more surface active due to the presence of methyl ester groups, which are more hydrophobic than carboxyl group (Schmidt, et al., 2015). Based on this, it was expected that the WPI-HMP conjugates would have a better emulsifying activity and produce emulsions with a smaller droplets size. Nevertheless, the results showed that the difference between the droplet size of the emulsions stabilized by the conjugates in the presence of LMP and HMP was

comparable. Einhorn-Stoll, et al. (2005) described that the emulsifying properties of protein-pectin conjugates were also determined by the compatibility of the protein and the polysaccharides. Proteins and polysaccharides with high compatibility will result in protein-polysaccharide conjugates with improved emulsifying properties. The authors also mentioned that the compatibility of the proteins and polysaccharides is different from protein to protein. Whey proteins were found to be compatible with pectin at any DE (Einhorn-Stoll, et al., 2005; Wang & Qvist, 2000). Nevertheless, these authors reported that the WPI-pectin conjugates had a slightly better emulsifying activity when pectin with a higher DE was used. Contrary to the finding of Einhorn-Stoll, et al. (2005), Schmidt, et al. (2016) reported that the DE did not influence the performance of the resulting conjugates, but continued by suggesting that the usage of LMP over HMP is more advisable since a higher yield was obtained from the incubation of WPI with the former. This finding is in line with the results obtained in this study. Emulsions stabilized by WPI-LMP and WPI-HMP conjugates exhibited a comparable droplet size which shows that the emulsifying activity of the conjugates containing dialyzed HMP and LMP was comparable.

The DE of pectin was found to have no influence on the heat stabilizing activity of the conjugates. Regardless of the DE of the pectin, the heat stability of WPI largely improved upon conjugation with pectin. The stabilizing activity of protein-polysaccharide conjugates was reported to be highly determined by the molecular weight of the polysaccharides (Shu, et al., 1996a). Polysaccharides with a minimum molecular weight of several kDa are required to have conjugates with improved heat stability. Polysaccharides with a higher molecular weight (3.5-24 kDa) had a better stabilizing activity than that with a much smaller molecular weight since the former was suggested to provide a greater steric stabilization (Shu, et al., 1996a). Moreover, these authors mentioned that in the range of the MW mentioned, the heat stability of the conjugates was comparable regardless of the molecular weight of the polysaccharide. The presence of these high molecular weight polysaccharides prevents the unfolded proteins to aggregate during heating via steric stabilization leading to the stabilization of protein and oil droplets (Liu, et al., 2013). For protein-polysaccharide conjugates, Kato (2002) and Dickinson (2008) proposed that polysaccharides with a minimum molecular weight of 10 kDa are needed to obtain conjugates with improved functionality. Both HMP and LMP had a significantly higher molecular weight (±100 kDa) which was sufficient to prevent protein

aggregation during heat treatment of the emulsions. Thus, the heat stability of the emulsions stabilized by the WPI-LMP and WPI-HMP conjugates might be governed more by their molecular weight than by their DE.

6.3.3.2 Viscosity of the emulsions

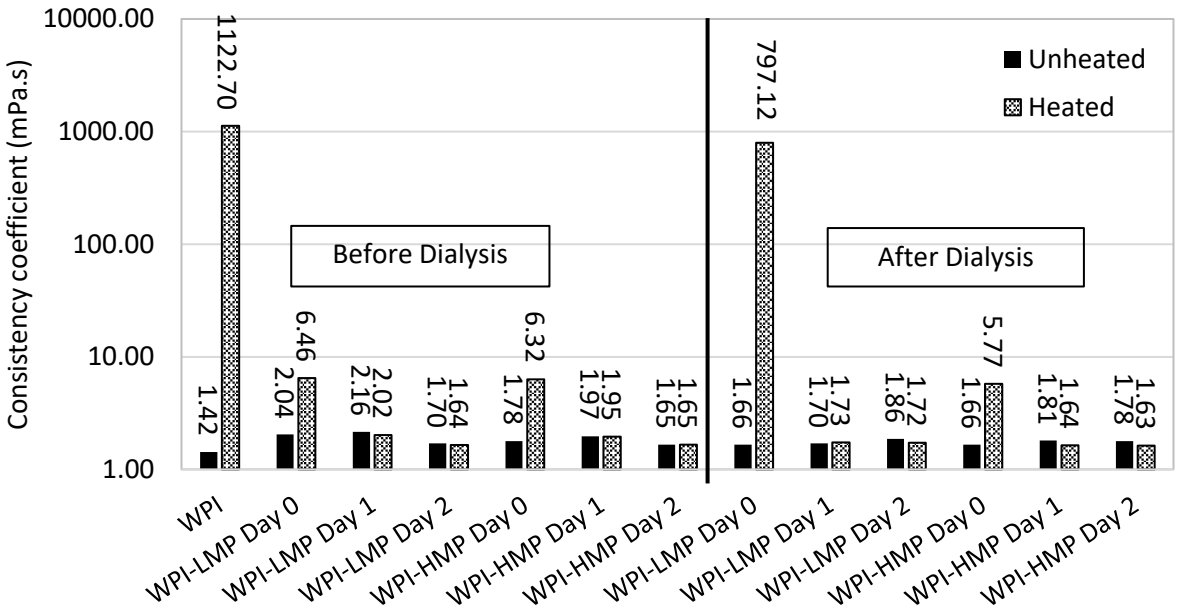


Figure 6.7. Consistency coefficients of the 10% (w/w) o/w emulsions stabilized by 0.5% of native WPI, WPI-pectin mixtures (WPI:pectin ratio 2:1, Day 0) or WPI-pectin conjugates (WPI:LMP ratio 2:1, Day 1-2) prepared with dialyzed and non-dialyzed pectin (HMP/LMP) before and after heating at 80^{o}C for 20 minutes at pH 6.5.

Viscosity can be used to evaluate the stability of emulsions against heat. The viscosity of the emulsions stabilized by the WPI, WPI-pectin mixtures, and WPI-pectin conjugates was evaluated. Figure 6.7 shows the consistency coefficient of emulsions stabilized by WPI, WPI-pectin mixtures and WPI-pectin conjugates prepared with the dialyzed and non-dialyzed pectins. Adding pectin in the emulsion can be seen to slightly improve the viscosity of the emulsions, which was expected. The emulsions stabilized by the conjugates prepared with non-dialyzed and dialyzed pectin, had a low viscosity and exhibited a Newtonian behaviour. Furthermore, sugar removal from the LMP and HMP seemed to have no noticeable effect on the viscosity of the emulsions. The DE did not influence the viscosity of the emulsions stabilized by the conjugates prepared with both dialyzed and non-dialyzed pectin. The results

might be due to the fact that the pectin concentration used in this study was low. Thus, no noticeable influence of pectin on the viscosity of the emulsions could be observed. At low concentration, the pectin molecules are suggested to be too far from each other to interact and to influence the viscosity of the system (Chan, et al., 2016; Guimaraes, Coelho Júnior, & Garcia Rojas, 2008).

After heat treatment, the emulsions stabilized by WPI and mixture of WPI and pectin underwent a change in their consistency. This observation confirmed the findings from the particle size measurements and from microscopic images, which showed that these emulsions underwent heat induced droplet aggregation and flocculation (Table 6.3). Flocculated droplets tend to have a higher viscosity due to the presence of water entrapped in between the flocculated droplets which increases the effective volume fraction of the particles (Dickinson, 1992). Furthermore, flocculated droplets exhibit shear thinning behaviour when the flocculated droplets are sheared (Fig. 6.8). In the case of emulsions stabilized by WPI-pectin conjugates, the emulsions exhibited a great thermal stability which was shown by the ability of the emulsions to maintain their consistency after heating. These phenomena were observed in conjugates prepared with dialyzed and non-dialyzed pectin.

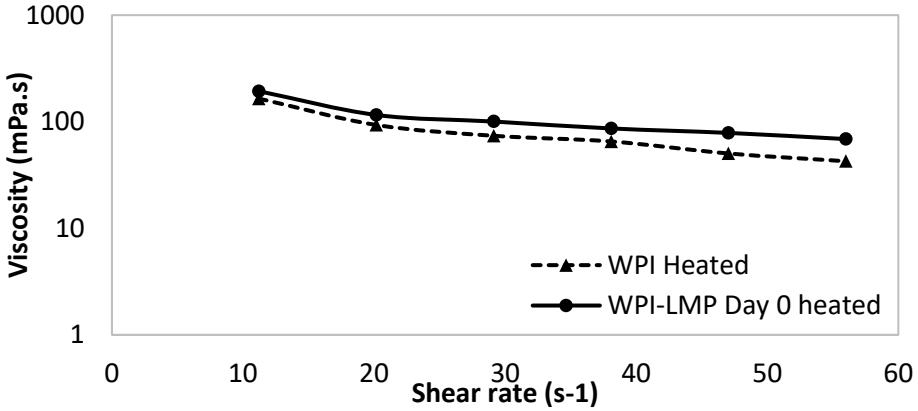


Figure 6.8. Apparent viscosity profile of heated 10% (w/w) o/w emulsions stabilized by 0.5% native WPI or WPI-LMP mixture (ratio 2:1) prepared at pH 5.0.

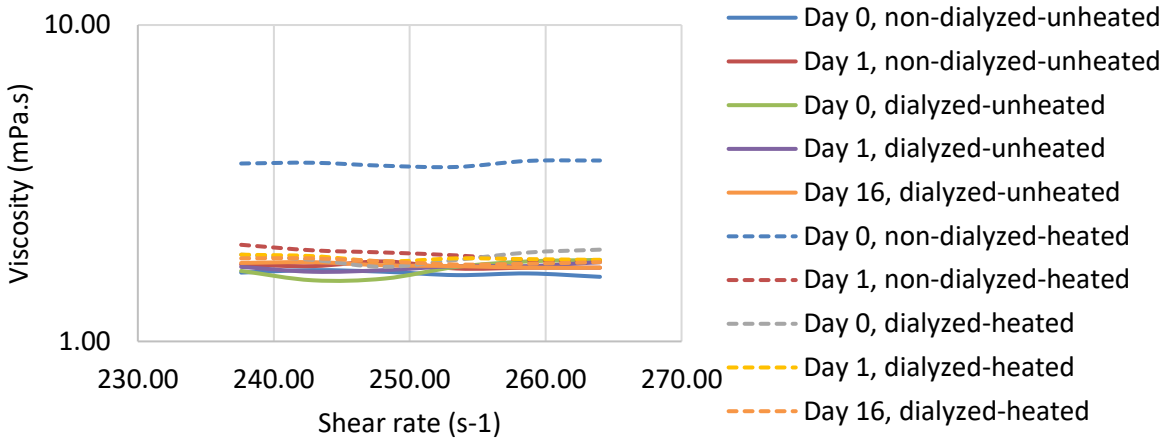


Figure 6.9 Viscosity profile of unheated and heated 10% (w/w) o/w emulsions stabilized by 0.5% WPI-HMP mixtures or by WPI-HMP conjugates (ratio 2:1) prepared at pH 5.0.

At pH 5.0, the emulsions stabilized by a WPI-HMP mixture and by WPI-HMP conjugates were able to retain their initial consistency after heating, which was in line with the results from droplet size measurements (Fig. 6.9). Only the emulsion stabilized by a WPI-HMP mixture prepared with non-dialyzed HMP underwent an increase in its viscosity after heating.

6.3.3.3 Electrophoretic mobility

The electrophoretic mobility (EM) of the emulsions was measured to study the stabilizing mechanism of the conjugates prepared with LMP and HMP. The EM of the emulsions was measured at pH 6.5 (pH of the emulsions) and at pH 5.0 (i.e. close to the IEP of the WPI). The bigger the EM of an emulsion, the stronger the electrostatic repulsion is, and thus the more stable the emulsion is expected to be.

Pectin is an anionic polysaccharide. Since methylation of the carboxyl group decreases the net charge density, pectin with a low degree of methylation tends to have a greater charge density than that with a higher degree of methylation (Harding, et al., 2017; Lapasin & Pricl, 1995). Therefore, in the presence of LMP, it was expected that the emulsion would be more negatively charged than in the presence of HMP. Table 6.5 shows the EM of emulsions stabilized by WPI, WPI-pectin mixtures, and WPI-pectin conjugates. At pH 6.5, the EM of emulsions stabilized by native WPI was lower than that of emulsions stabilized by WPI-pectin conjugates. Furthermore, the EM of emulsions stabilized by WPI in the presence of LMP was higher than in the presence of HMP ($p<0.05$), as expected. At pH 6.5, the EM of emulsions

stabilized by WPI-pectin mixtures (at Day 0) was comparable to that stabilized by WPI only. Due to dry heat treatment, the pectin becomes covalently linked to WPI. Hence, upon adsorption of WPI to the surface of the oil droplets, pectin was also adsorbed. Consequently, the overall charge density of the oil droplets was affected by the presence of pectin. Furthermore, the conjugation of WPI-LMP and WPI-HMP took place on the positively charged amino groups of the protein, which reduced the number of positive charges of the protein (Schmidt, et al., 2016). Without dry heat treatment, it was expected that pectin would not be adsorbed at the surface of oil droplets. Thus, the EM of the emulsions stabilized by WPI-pectin mixtures would be comparable to that of emulsions stabilized by WPI only. Nevertheless, a weak interaction of protein and polysaccharide at around neutral pH is possible, albeit very limited and reversible (Dickinson, 2008; Einhorn-Stoll, et al., 1996).

Table 6.5. Electrophoretic mobility (mean values and standard deviations, expressed in µm.cm/V/s) of emulsions stabilized by WPI, WPI-pectin mixtures and WPI-pectin conjugates at pH 6.5 and pH 5.0.

Sample	Treatment	Incubation time (days)	pH 6.5	pH 5.0
WPI			$-2.61\pm0.07^{1,2}$	-0.33 ± 0.02^{1}
WPI-LMP	Non-dialyzed	0	-2.75 ± 0.03^{aA2}	-1.97 ± 0.02^{aA2}
		1	-3.82 ± 0.08^{bA}	-2.64 ± 0.05^{bA}
	Dialyzed	0	-2.83 ± 0.11^{aA2}	-1.98 ± 0.05^{aA2}
		1	-3.28 ± 0.14^{bA}	-2.16 ± 0.04^{bA}
WPI-HMP	Non-dialyzed	0	-2.50 ± 0.07^{aB1}	-1.64 ± 0.01^{aB3}
		1	-2.92 ± 0.06^{bB}	-2.23 ± 0.06^{bB}
	Dialyzed	0	$-2.71\pm0.14^{aA1,2}$	-1.65 ± 0.08^{aB3}
		1	-3.16 ± 0.12^{bA}	-2.17 ± 0.08^{bA}

^{a,b} are used to compare between day 0 and day 1 within the same group, for example WPI-LMP conjugates, non-dialyzed day 0 and 1.Means in the same column and group followed by different lowercase letters are significantly different ($p<0.05$).

^{A,B} are used to compare dialyzed and non-dialyzed conjugates which are prepared with the same pectin and the same incubation time, for example, between non-dialyzed and dialyzed HMP-LMP mixture day 0, and between non-dialyzed and dialyzed HMP-LMP conjugates Day 1. Means in the same column followed by different uppercase letters are significantly different ($p<0.05$).

^{1,2} are used to compare the emulsions stabilized by WPI, WPI-LMP mixture Day 0 (dialyzed and non-dialyzed), and WPI-HMP mixture day 0 (dialyzed and non-dialyzed).Means in the same columns followed by different numbers are significantly different ($p<0.05$).

pH 5.0 is known to be close to the IEP of WPI. Thus, it was reasonable that the emulsions stabilized by WPI had an EM value close to zero (-0.3 µm.cm/V/s). At a pH around the IEP of the protein, the net charge density of the emulsions will be close to zero, leading to the production of an unstable emulsion. At pH 5.0, it was noted that the EM of emulsions stabilised by WPI-pectin mixtures and conjugates exhibited the same trend as that observed at pH 6.5, but with a lower value. At nearly neutral pH, the carboxyl groups of pectin are negatively charged, as the pH is lowered, pectin becomes more protonated, which shifts the EM to a lower value. Compared to the EM of emulsions stabilized by WPI only, the EM of emulsions stabilized by WPI in the presence of pectin was higher which explains the stability of emulsions around the IEP of WPI (Neirynck et al., 2004). The IEP of the emulsions stabilized by a mixture was significantly higher than that of an emulsion stabilized by WPI only due to the presence of pectin bound to the WPI via electrostatic interaction ($p<0.05$). Furthermore, the IEP of the emulsions stabilized by the mixture of WPI and LMP was higher ($p<0.05$) than that of an emulsion stabilized by a mixture of WPI-HMP, which could also be attributed to the higher amount of LMP present at the surface of the oil droplets. It has been reported that electrostatic interaction between β-lactoglobulin and LMP results in a higher amount of complexation as compared to HMP (Girard, et al., 2002). In the case of emulsions stabilized by the conjugates, in addition to the covalently linked pectin, there was additional pectin linked to the WPI via electrostatic interaction. Thus, a higher EM was observed for emulsions stabilized by the conjugates. The influence of the DE can also be observed in the emulsions stabilized by the conjugates. The conjugates prepared with LMP possessed a higher EM than that prepared with HMP. Furthermore, the EM measurements at pH 5.0 showed that conjugation of LMP to WPI shifted the IEP of the WPI as it was reported in the previous study (Setiowati, et al., 2017).

6.3.3.4 Accelerated creaming stability of the emulsions

Table 6.6. Creaming velocity (expressed in mm/day) of 10% (w/w) o/w emulsions stabilized by 0.5% WPI, WPI-pectin mixtures or WPI-pectin conjugates prepared with dialyzed and non-dialyzed LMP and HMP pectins at pH 6.5 measured at 1200 g for 1 hour.

Sample	Incubation time (day)	Non-dialyzed		Dialyzed	
		Unheated	Heated	Unheated	Heated
WPI		162.5 ± 26.3	4197 ± 3076		
WPI-LMP	0	251.3 ± 6.8^{aA}	3738.0 ± 339.5^{B}	253.1 ± 0.2^{aA}	1939.1 ± 277.8^{B}
	1	105.1 ± 0.7^{bA}	108.1 ± 0.7^{A}	114.2 ± 1.1^{bA}	120.9 ± 0.1^{A}
	2	102.6 ± 0.2^{bA}	105.9 ± 0.5^{A}	103.6 ± 0.7^{cA}	104.5 ± 0.3^{A}
WPI-HMP	0	239.3 ± 1.5^{aA}	1969.6 ± 908.6^{B}	186.1 ± 0.4^{aA}	850.2 ± 62.7^{B}
	1	99.4 ± 0.1^{bA}	100.4 ± 0.1^{B}	105.1 ± 0.1^{bA}	106.4 ± 0.7^{A}
	2	98.6 ± 1.7^{bA}	100.2 ± 0.8^{B}	90.3 ± 2.6^{cA}	94.5 ± 0.7^{A}

^{a,b} Means in the same column followed by different lowercase letters are significantly different (P<0.05).
^{A,B} Means in the same row followed by different uppercase letters are significantly different (p<0.05).

Table 6.6 shows the creaming velocity of emulsions stabilized by WPI and WPI-pectin mixtures and conjugates. It can be seen that the creaming stability of unheated emulsions stabilized by WPI and by a mixture of WPI and pectin was poor. Only the unheated emulsions which were stabilized by WPI-pectin conjugates exhibited a great stability against creaming. The presence of unconjugated pectin apparently decreased the creaming stability of the emulsion. The lower stability against creaming of the emulsions stabilized by a mixture of WPI and pectin compared to that stabilized by WPI only might be due to droplet flocculation by the presence of pectin present in the aqueous phase, which may be either due to bridging or depletion flocculation. It was also found that the emulsions stabilized by WPI-pectin conjugates prepared with non-dialyzed pectin (LMP/HMP) had a lower creaming rate than that prepared with dialyzed pectin ($p<0.05$). This is reasonable since the emulsions stabilized by the conjugates prepared with dialyzed pectin had a bigger droplet size. Based on Stokes' law, emulsions with bigger droplets will cream faster than those with smaller droplets (Dickinson, 1992). In addition, the presence of sugar in the non-dialyzed pectin might also explain this phenomenon. It has been reported that the presence of sucrose in an emulsion stabilized by β-lactoglobulin improved the stability against droplet flocculation upon storage at constant temperature (Kim, et al., 2003). The authors explained that this was due to the ability of sucrose to stabilize the conformation of the adsorbed protein to prevent further surface

denaturation which can induce droplet flocculation and coalescence. The emulsions stabilized by WPI and by a mixture of WPI and pectin were unstable against heat induced droplet aggregation and thus the creaming velocity increased dramatically after heating. In contrast, all emulsions stabilized by the WPI-pectin conjugates exhibited an excellent stability against heat as there was only a minor change in the creaming velocity of the emulsions after heating.

As for the effect of the degree of esterification, in general the results show that the unheated and heated emulsions stabilized by the conjugates had a better stability against creaming in the presence of HMP ($p<0.05$). Based on the results of EM measurements, it was expected that the emulsions stabilized by WPI-LMP conjugates would have a better stability against creaming than the emulsions stabilized by WPI-HMP conjugates since the former were more negatively charged than the latter and hence were expected to have a lower aggregation tendency. However, the creaming velocity measurements showed that the emulsions stabilized by WPI-HMP conjugates were more stable against creaming. Therefore, it seems that in this case, steric stabilization had a bigger role than charge repulsion in stabilizing the emulsions. It has been reported that when combined with protein, LMP induced more bridging than HMP by interacting with more proteins due to its higher charge density (Jones, Decker, & McClements, 2010). In this case, LMP might interact with the adsorbed WPI from different droplets through weak electrostatic interaction which can cause the droplets to be in a close vicinity leading to droplet flocculation. This might explain the better creaming stability exhibited by emulsions stabilized by WPI in the presence of HMP. Similar results were also reported by (Einhorn-Stoll, et al., 2005). Regarding the heat stability of the emulsions, there was no influence of DE as the emulsions stabilized by the WPI-LMP and WPI-HMP conjugates were able to maintain their creaming velocity after heat treatment.

Table 6.7 shows the creaming velocity of emulsions stabilized by a WPI-HMP mixture and by WPI-HMP conjugates at pH 5.0. The results indicate that the emulsions were stable at pH 5.0, which is around the IEP of WPI. Without dry heat treatment, the creaming velocity of the emulsions stabilized by the WPI-HMP mixture prepared using non dialyzed HMP increased after heat treatment due to the presence of flocculated droplets. In contrast, the heated emulsion was relatively stable against creaming when the mixture was prepared using the dialyzed HMP. There was only a slight increase in the creaming velocity of the emulsions after heating. Nevertheless, their creaming velocity was still significantly higher than that of

stabilized by the conjugates. The same phenomenon was observed in emulsions stabilized by WPI in the presence of LMP (data not presented). Longer incubation times seemed to improve the creaming velocity of the emulsions. Incubation of the WPI-HMP conjugates for 16 days produced emulsions with the lowest creaming stability. At this pH, it was once again observed that pectin with a higher DE provided a better creaming stability than pectin with lower DE.

Table 6.7. Creaming velocity (expressed in mm/day) of 10% (w/w) o/w emulsions stabilized by a 0.5% WPI-HMP mixture or WPI-HMP conjugates at pH 5.0, prepared with dialyzed and non-dialyzed pectin measured at 1200 g for 1 hour.

Sample	Incubation time (day)	Non-dialyzed		Dialyzed	
		Unheated	Heated	Unheated	Heated
WPI-HMP	0	254.7 ± 1.2^{aA}	710.2 ± 5.5^{B}	210.3 ± 0.2^{aA}	218.9 ± 0.7^{B}
	1	158.2 ± 0.5^{bA}	162.8 ± 0.3^{A}	160.2 ± 1.3^{bA}	163.9 ± 1.5^{B}
	16			133.0 ± 0.2^{cA}	136.3 ± 0.8^{B}

^{a,b} Means in the same column followed by different lowercase letters are significantly different ($p<0.05$).

^{A,B} Means in the same row followed by different lowercase letters are significantly different ($p<0.05$).

6.4 Conclusions

The results showed that the sugar present in pectin was successfully removed by dialysis. Dry heated conjugates prepared with dialyzed HMP produced significantly less brown pigment which indicated that the dialysis was able to remove the dextrose from the HMP. Furthermore, it also shows that the dextrose present in HMP was also involved in the Maillard reaction during dry heat treatment of WPI and HMP. On the other hand, sucrose did not have a great impact during dry heat treatment of WPI with LMP. The results obtained in this study generally revealed that the DE did not have a significant influence on the emulsifying activity of WPI-pectin conjugates and heat stability of the emulsions stabilized by the conjugates. However, the creaming stability of the emulsions was influenced by the DE of the pectin used. Despite of having a lower EM, the emulsions stabilized by WPI-HMP conjugates provided a better stability against creaming than those with a lower DE. This indicated that steric stabilization might play a more important role than electrostatic repulsion. Nevertheless, the role of electrostatic repulsion in stabilizing the emulsions cannot be completely ruled out either. Contrary to the results obtained by Neirynck, et al. (2004), the WPI-HMP conjugates exhibited

excellent emulsifying activity at a pH close to the IEP of WPI when the HMP used to prepare the conjugates was dialyzed. Hence, the poor emulsifying properties shown by WPI-HMP conjugates as reported previously were due to advanced Maillard reaction between dextrose and WPI which took place at longer incubation times. Furthermore, the emulsions also exhibited excellent heat stability.

It is necessary to eliminate the sugars when evaluating the influence of DE on the performance of WPI-pectin conjugates. Without performing dialysis, the phenomena observed are most likely due to a combined effect of sugar, pectin, and protein present in the emulsion, which is undesirable. By removing the sugar from the pectin, its influence was eliminated or limited. Thus, the results truly reflect the influence of DE.

Based on this study, HMP is more advisable than LMP if the stability of the products against creaming is of great concern since the former offers better stability against creaming over time. Nevertheless, both HMP and LMP offer comparable emulsifying activity and heat stability. For its application especially on the industrial scale, it is not necessary to perform dialysis on the standardized pectin as long as the dry heat treatment is performed for a sufficiently short time. This is due to the fact that the conjugates prepared with non-dialyzed pectin also exhibited a great functionality which was comparable with the functionality of the conjugates produced with dialyzed pectin. This will save the time invested for the preparation of the conjugates. For an application in which longer dry heat treatment is required or the dry heat treatment is performed at much higher temperature, LMP is more preferable. As it does not contain low molecular weight reducing sugars, exposing a mixture of WPI and LMP to a longer period of dry heat treatment without performing dialysis will not have an adverse impact on the functionality of the conjugates produced.

CHAPTER 7

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

7.1 Conclusions

The heat stability of milk proteins, especially whey proteins, is critical for the milk industry. Heating of milk proteins at high temperature causes protein denaturation and aggregation. In dairy industry, protein denaturation and aggregation create problems by causing fouling in heat exchanger plates which reduces the heat transfer efficiency. Milk proteins consist of caseins and whey proteins. The latter are known to be very heat labile. Nevertheless, whey proteins have excellent and various functionalities. They are known as good emulsifying, gelling, as well as foaming agents. However, the functionality of whey proteins is highly influenced by their heat stability. When they are exposed to heat, protein aggregation may take place leading to the reduction of their solubility. Since the functionality of whey proteins is determined by their solubility, protein aggregation will have a negative impact. This is especially problematic as heat treatment in food industry is almost inevitable to prolong the shelf life of food products. As heat treatment is commonly performed at temperatures above the denaturation temperature of whey proteins, the heat stability of whey proteins is a factor which limits their application in food industry. Therefore, a lot of research to improve the heat stability of whey proteins has been performed in the past, and is still being performed nowadays.

From the literature study presented in Chapter I, it is known that the heat stability of whey proteins is influenced by different factors, such as electrolytes, pH, and temperature. Upon heating at temperatures higher than the denaturation temperature of the whey proteins, they become (at least partly) unfolded, exposing hydrophobic groups and reactive amino groups of the proteins which were previously buried. This phenomenon will then trigger the association or interaction of the unfolded proteins resulting in insoluble aggregates. The reactions involved in the heat induced denaturation and aggregation of whey proteins have been well studied and identified. The aggregation of the unfolded protein has been mentioned to take place via covalent interactions (disulphide bonds), hydrophobic interactions, as well as ionic, and Van der Waals interactions (to some extent). To improve the heat stability of whey proteins it is necessary to prevent their unfolding and/or the association of the unfolded whey proteins. Numerous methods have been proposed by researchers to improve the heat stability of whey proteins, such as enzymatic modification, addition of a thiol group blocking agent, or combining whey proteins with other biopolymers.

This study aimed to improve the heat stability of whey proteins via dry heat treatment with polysaccharides, which may result in the formation of WPI-polysaccharide conjugates via Maillard type reactions. Whey protein isolate which had a high concentration of protein (97.7%) and a low concentration of lactose (0.5-1%) was used in this study. In addition, the WPI contained some cations such as K^+ (0.08%), Na^+ (0.40%), Ca^{2+} (0.08%), and Mg^{2+} (0.013%) (Neirynck, et al., 2004). The WPI was dry heat treated with HMP and LMP containing 90.5 and 89.6% of dry matter, respectively. It was reported previously by Neirynck, et al. (2004) that the LMP contained 0.10%, 3.37%, 0.08%, and 0.01% (w/w) of K^+, Na^+, Ca^{2+}, and Mg^{2+}, respectively. On the other hand, the HMP contained 0.04%, 0.24%, 0.22%, and 0.01% (w/w) of K^+, Na^+, Ca^{2+}, and Mg^{2+}, respectively. Furthermore, it has to be mentioned that according to the manufacturer, 5% of sucrose and 40% of dextrose have been added to the LMP and HMP, respectively. The presence of these non-reducing (sucrose) and reducing (dextrose) sugar was confirmed by gas chromatography analysis. It was expected that improving the heat stability of whey proteins through dry heat treatment of whey protein with pectin would broaden their application in the food industry.

Generally, based on the results of our study, dry heat treatment of WPI and pectin indeed induced the formation of covalent complexes, as it was illustrated by the reduced amount of free amino groups, the emergence of high molecular weight components in SDS-PAGE and the decreased mobility of whey proteins as measured by diffusion-NMR. These conjugates were formed by covalent attachment of the LMP to WPI through Maillard type reactions during the dry heat treatment. As conjugation largely affected the diffusion behaviour of the proteins, but hardly changed the diffusivity of the pectin, it follows that complexes typically involve only 1 pectin molecule, chemically bound to either 1 or more protein molecules. As far as time effects were considered, the formation of the conjugates was found to be rapid during the first two days of dry heat treatment and continuously increased at a slower rate upon extending the dry heat treatment to 16 days (Chapter 5). Higher conjugation yields were also obtained when the concentration of LMP was increased.

The presence of sugar, in particular dextrose, was reported to interfere with the formation of the conjugates during dry heat treatment. The HMP and LMP used in this study had been standardized by adding 40% of dextrose and 5% of sucrose, respectively. It was observed that advanced Maillard reaction between dextrose present in the HMP with WPI took place at

longer dry heat treatment time which led to browning and polymerization of the protein. As a consequence, a reduction of the functionality of the WPI was observed. Therefore, to obtain WPI-polysaccharide conjugates with the desired properties, it is very important to avoid advanced Maillard reaction. With regard to the influence of DE, previous research indicated a pronounced difference between LMP and HMP. However, our research indicated that the DE hardly played a role on the functionality of the WPI-pectin conjugate provided that the added sugars were previously removed (Chapter 6).

In O/W emulsion systems, the whey protein-pectin conjugates successfully improved not only the emulsifying activity, but most importantly, also the heat stability of the WPI (Chapter 2) as well as WPI stabilized emulsions (Chapter 3): the solutions of WPI-LMP conjugates were stable against heat and no visible aggregates were observed in the solutions after heating. Protein solubility measurements showed that there was almost no change in the solubility of the conjugated WPI after heating.

The heat stability of the unadsorbed WPI has been reported to play an important role in the heat stability of WPI stabilized emulsions. As heat induced protein aggregation may cause the aggregation of unadsorbed WPI in the aqueous phase with the adsorbed proteins from two or more oil droplets, it follows that oil droplets may start aggregating, whereby the unadsorbed proteins may act as a glue in between the oil droplets (Fig 7.1 a). Due to the strong electrosteric repulsion by binding with pectin, this phenomenon was largely prevented in the presence of WPI-LMP conjugates (Fig 7.1 b).

The WPI-LMP conjugates stabilize the oil droplets mainly through the same mechanism. QCM-D measurements (Chapter 4) indeed revealed that WPI-LMP conjugates formed a thick viscoelastic layer (Fig 7.2 a) on the surface of the oil droplets. This layer prevents the oil droplets to be in close vicinity, thus reducing the contact between oil droplets or between proteins and inhibiting droplet and protein aggregation. This layer was also believed to be responsible for the stability of the emulsions against creaming over time. Compared to WPI-LMP conjugates, WPI formed a relatively less viscoelastic and thinner layer (Fig 7.2 b), except at pH conditions close to the IEP, where the layer tended to be more rigid (Fig 7.2 c). Furthermore, the functionality of WPI is especially poor around its IEP, i.e. around pH 5.0. At this pH, WPI exhibits a low protein solubility and poor heat stability. Our study revealed that

dry heat treatment of WPI and LMP was very effective to improve the heat stability of WPI and the emulsifying activity of WPI stabilized emulsions around its IEP which was attributed to the shift of IEP of the protein upon binding with the anionic pectin molecules. Experimental measurements indeed indicated that the IEP of WPI was shifted to a lower pH value upon dry heat treatment with LMP.

Droplet size measurement indicated that the emulsifying activity of the WPI-LMP conjugates was significantly improved compared to that of native WPI, especially around the proteins' IEP: the conjugation of WPI with LMP enabled the production of emulsions with a smaller droplet size. In Chapter 3, it was found that the droplet size of the emulsions decreased considerably when the WPI-LMP mixture was dry heat treated for 1 day and continuously decreased slightly as the dry heat treatment time was prolonged. The same trend was also observed when the concentration of LMP in the conjugates was increased. These results indicated that the emulsifying activity of the WPI-LMP conjugates might be related to the yield of the WPI-LMP conjugation. It was found that conjugates with a higher yield of conjugation (higher degree of interaction) resulted in a higher emulsifying activity. Moreover, it was observed that the emulsions stabilized by WPI-LMP conjugates were highly stable against heat induced droplet aggregation when heated at 80 and 120^{o}C. Whereas typically quite long dry heat incubation times are proposed (ranging from days to weeks), overnight dry heat treatment (24 hours) was shown to be sufficient to greatly improve the heat stability of WPI. After 1 day of dry heat treatment, approximately 27% of the WPI was covalently bound to LMP. It was interesting that despite the fact that WPI-LMP conjugates which were dry heat treated for 1 day had a lower yield than those dry-heat treated for 16 days, the heat stability of these two conjugates was comparable. Thus, it seemed that the heat stabilizing activity of the WPI-LMP conjugates was not influenced by the yield or degree of interaction to a large extent. On the other hand, dry heating of WPI in the absence of LMP did not significantly influence the functional properties of WPI. This showed that the improvement of the heat stability and emulsifying activity of the conjugated WPI solely due to the presence of WPI-LMP conjugates.

Simple mixing of WPI with LMP improved the emulsifying activity and heat stability of WPI, provided that the environmental conditions favour electrostatic interaction (i.e. pH that limits electrostatic repulsion (close to IEP), as well as low electrolyte concentration) (Chapter 3).

Even under favourable conditions, the pectin concentration was preferably (at least) equal to the protein concentration (WPI to LMP ratio of 1:1). The performance of these WPI-pectin complexes was highly influenced by the concentration of LMP, pH, and presence of NaCl. At environmental conditions where the electrostatic interaction was not favourable, the emulsions stabilized by mixtures of WPI and LMP were not able to withstand heat treatment and an increase in the droplet size of the emulsions was observed. Furthermore, the consistency of the emulsions changed due to the presence of the droplet aggregates.

In addition, despite of having a higher degree of interaction (yield), emulsions stabilized by WPI-LMP electrostatic complexes (formed at pH around the IEP of WPI) were found to be less stable against creaming and heat than that stabilized by WPI-LMP conjugates (Chapter 6). Furthermore, the former had a lower emulsifying activity than the latter. Therefore, it seemed that the nature of the interaction between WPI and LMP was an important factor which determines the heat stability and emulsifying activity of the WPI-LMP conjugates. The results of our study also indicated that covalent interaction between WPI and LMP achieved via dry heat treat treatment was stronger than that achieved via electrostatic interaction.

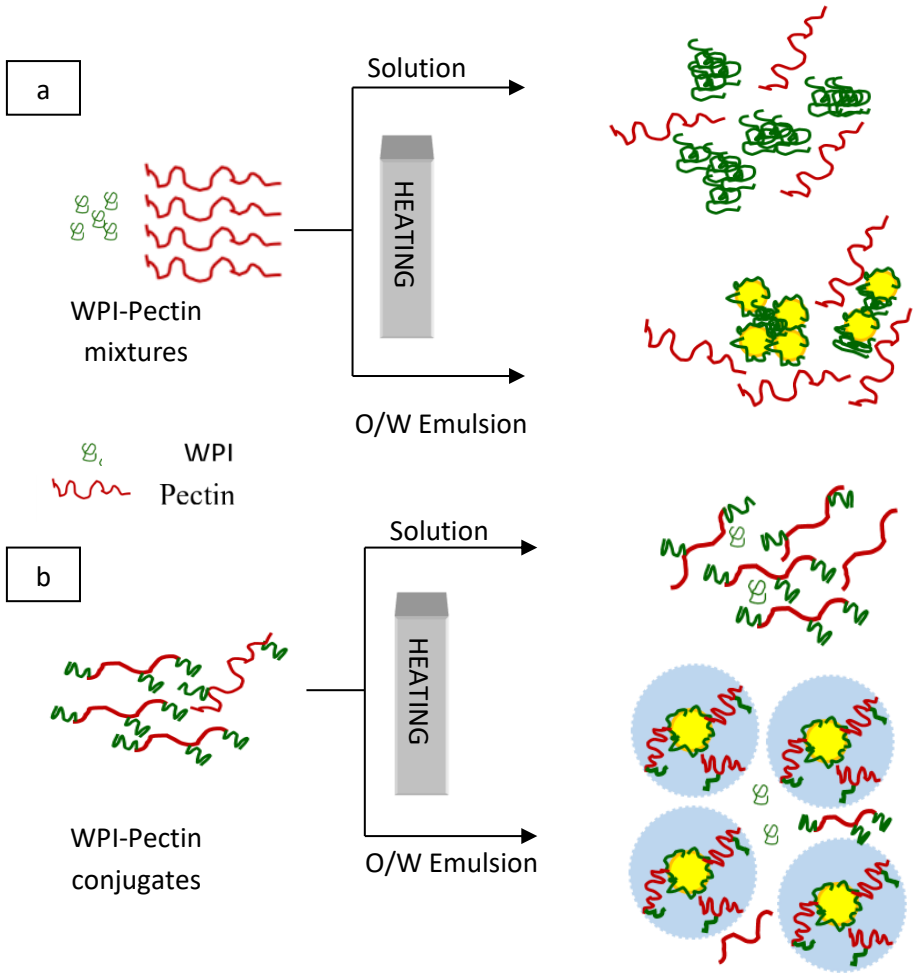


Figure 7.1. Heat stabilizing activity of WPI-pectin mixtures (a) in solution and O/W emulsion as compared to that of WPI-pectin conjugates (b)

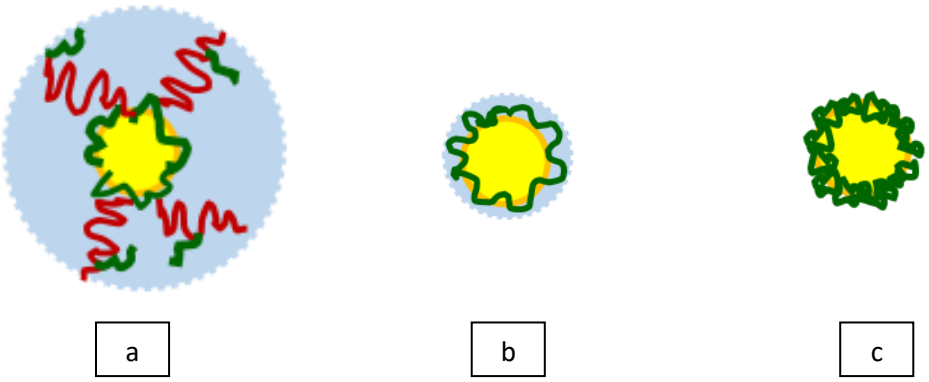


Figure 7.2. Schematic image of adsorbed layer of WPI-LMP conjugates (a) and WPI on the surface of oil droplets at pH 6.5 (> IEP) (b) and pH 5.0 (≈ IEP) (c).

7.2 Future perspectives

This doctoral study was aimed to improve the heat stability of whey proteins through dry heat treatment with pectin. The results clearly showed that this method was successful in improving the heat stability as well as emulsifying activity of the whey proteins. In this study, dry heat treatment of WPI and pectin mixtures for one day was required to improve the heat stability of whey proteins and whey protein stabilized emulsions. It might be interesting to study the influence of processing conditions (such as: temperature and relative humidity) in order to further reduce the required time. Furthermore, the possibility of replacing pectin with sugars could also be investigated. This is due to the fact that reducing sugars are more reactive and hence shorter reaction times may be possible.

Further studies to clarify the stabilization mechanism exhibited by the WPI-pectin conjugates are needed especially at a molecular level. Since a limited amount of WPI-pectin conjugates was already sufficient to stabilize the protein against heat, it might be interesting to study the adsorption of the WPI-pectin conjugates to the surface of oil droplets and to identify the composition of the adsorbed layer. This could provide information on the minimum amount of pectin needed at the interface to ensure heat stability. Determination of the surface activity of the conjugates can also be performed, accompanied by evaluation of the chemical structure of the WPI-pectin conjugates as influenced by dry heating using, for instance, FTIR. Furthermore, in this study the WPI-LMP conjugates were used without any purification to remove the unreacted WPI and LMP. Therefore, it would be interesting to purify the conjugates and evaluate their performance in the absence of unreacted WPI and pectin.

Nowadays, the interest in creating clean-label emulsions (i.e. emulsion without synthetic emulsifiers) is growing. Due to their excellent emulsifying activity and heat stability, WPI-pectin conjugates have a big potency to be used as natural emulsifiers to replace synthetic emulsifiers. Future applications of WPI-pectin conjugates in double emulsions and encapsulation of functional compounds are also worth to be evaluated. As the emulsions prepared in this study are quite diluted, it might be good to evaluate the performance of the WPI-pectin conjugates in systems containing a high amount of oil and higher concentration of protein. In addition, the functionality of whey proteins has been known to be influenced by the presence of cosolvents and cosolutes. Foods products normally contain different kinds of

ingredients such as electrolytes, sugar, flavourings, and colouring agents. Therefore, it would be interesting to study the performance of the conjugates in the presence of different cosolutes such as in the presence of a high amount of sugar or electrolytes.

The application of WPI-pectin conjugates in the production of concentrated milk and infant milk are also interesting to be investigated. For the former, it might also be interesting to study the performance of the conjugates in a system which contains casein, such as in skim milk. It is expected that the presence of conjugates will prevent the denaturation and aggregation of the whey proteins as well as their interaction with κ-casein during heat treatments and thus will prevent fouling. Besides protein, skim milk also contains lactose which is a reducing sugar. Therefore, by exposing skim milk to heat, Maillard reaction between the milk proteins and lactose present in skim milk could be induced. This can be performed by for instance dry heat treatment of skim milk powder (SMP) or spray drying of the skim milk. The latter is normally involved in the production of skim milk powder (SMP). During spray drying, the skim milk droplets are exposed to hot air for a certain period of time. This process might be modified to induce the formation of milk protein-lactose conjugates which is expected to improve the heat stability of milk proteins. This technique has the advantage that it does not require chemicals and polysaccharide addition from external sources. Nevertheless, a good process control is required since lactose is more reactive than high molecular weight polysaccharide and thus prone to advanced Maillard reactions.

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2010 - 2012 : Master of Science in Food Science (InterUniversity Programme Master of Science in Food Technology)
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2013 - present : Doctor in Applied Biological Sciences: Food Science and Nutrition
Faculty of Bioscience Engineering, Ghent University, Belgium

Awards and grants

October 2017 : Young Scientist Awards, The 10th NIZO Dairy Conference, Arnhem (NIZO 2017), The Netherlands

July 2013 : Selected as LPDP (Indonesian Endowment Fund for Education) awardee

September 2010 : Received MSc. Scholarship from VLIR

January 2009 : Grant from Lineaus Palme project to perform research in Chalmers University, Sweden for 6 months.

Publications

- **International peer-reviewed articles (A1 Journal)**
  - **Setiowati, A. D**., Saeedi, S., Wijaya, W., & Van der Meeren, P. (2017). Improved heat stability of whey protein isolate stabilized emulsions via dry heat treatment of WPI and low methoxyl pectin: Effect of pectin concentration, pH, and ionic strength. *Food Hydrocolloids, 63*, 716-726.

- **Setiowati, A. D.**, Vermeir, L., Martins, J., De Meulenaer, B., & Van der Meeren, P. (2016). Improved heat stability of protein solutions and O/W emulsions upon dry heat treatment of whey protein isolate in the presence of low-methoxyl pectin. *Colloids and Surfaces A: Physicochemical and Engineering Aspects, 510*, 93-103.
- Wijaya, W., Patel, A. R., **Setiowati, A. D**., & Van der Meeren, P. (2017). Functional colloids from proteins and polysaccharides for food applications. *Trends in Food Science & Technology*, 68, 56-69.

- **Manuscripts under preparation for submission**
  - **Setiowati, A. D**., Rwigamba. A., & Van der Meeren, P. The influence of degree of esterification on the emulsifying activity of Whey Protein-Pectin conjugates and on the heat stability of the emulsions stabilized by these conjugates
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  - **Setiowati, A. D**. & Van der Meeren, P. Heat stability of whey proteins and whey proteins-polysaccharides conjugates formed via dry heat treatment.

- **Oral presentations at conference and Workshop**
  - **Setiowati, A.D.** Improving Heat Stability of Whey Protein Isolate, Ghent, Belgium. The 21st National symposium on Applied Biological Sciences (NSABS 2016), Antwerp, Belgium.
  - **Setiowati, A.D.** Improved heat stability of whey protein isolate stabilized emulsions via dry heat treatment of WPI and low methoxyl pectin: Effect of pectin concentration, pH, and ionic strength. The 1st Innovation in Food Science and Technology conference (2017), Erding, Germany.
  - **Setiowati, A.D.** Dry heat treatment of whey protein isolate with low methoxyl pectin to improve heat stability of protein in solution and O/W emulsion. The 10th NIZO Dairy Conference, Arnhem (NIZO 2017), The Netherlands.

- **Poster presentation at conference**
  - **Setiowati, A. D**., Saeedi, S., & Van der Meeren, P. Dry heat whey protein-pectin conjugates largely improve the heat stability of protein stabilized O/W emulsions. 29th Conference of the European Colloid and Interface Society (Ecis 2015), Bordeaux, France.
  - **Setiowati, A. D**., Saeedi, S., & Van der Meeren, P. Improved Heat Stability of Whey Protein Isolate Stabilized Emulsions by Conjugation with Low Methoxyl Pectin using Dry Heat Treatment. 15th European Student Colloid Conference (2015), Krakow, Poland.
  - **Setiowati, A. D**. & Van der Meeren, P. Improved heat stability of whey protein isolate-stabilised emulsions by conjugation with Low Methoxyl Pectin through dry heat treatment. 16th edition of the Food Colloids conference (2016), Wageningen, The Netherlands.

Master student supervision

- Serveh Saeedi (2014/2015). Heat Stability Evaluation of Oil-in-Water Emulsions Stabilized by Whey Protein Isolate-Low Methoxyl Pectin Dry Heat Conjugates
- Alexis Rwigamba (2016/2017). Influence of Pectin Composition On Heat Stabilizing Effect Of Protein-Polysaccharides Dry Heat Conjugates

Doctoral training program (DTP) related to research

- Particle Characterization training (BePCis)
- Rheology of Colloidal Systems
- QCM-D training: Introduction to QCM-D
- QCM-D training: Data processing